FACTORS AFFECTING THE FRUITING OF OPHIOBOLUS GRAMINIS SACC.

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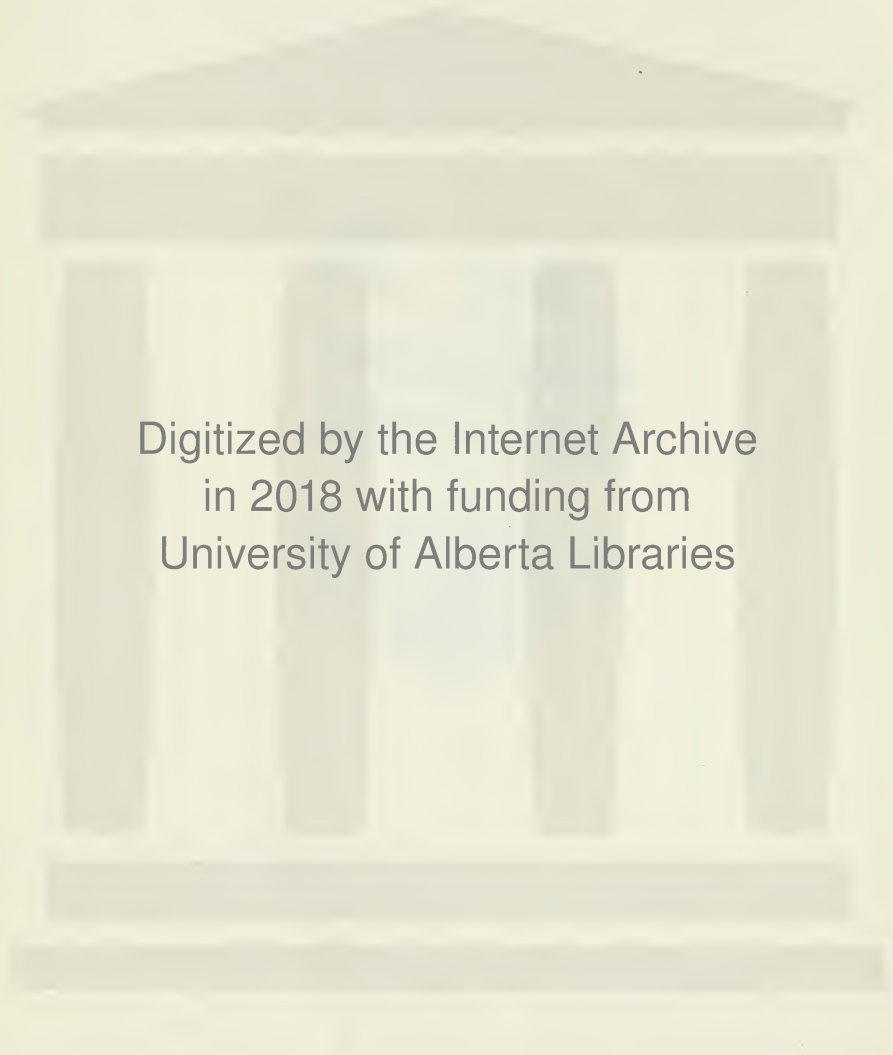

U N I V E R S I T Y O F A L B E R T A

FACULTY OF AGRICULTURE

The undersigned hereby certify that they have read and recommend to the Committee on Graduate Studies for acceptance a thesis on "Factors affecting the fruiting of Ophiobolus graminis Sacc.", submitted by J. D. Gilpatrick, B.Sc. (Agr.) in partial fulfilment of the requirements for the degree of Master of Science.

FACTORS AFFECTING THE FRUITING OF *OPHIOBOLUS GRAMINIS* SACC.

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A THESIS
submitted to the University of Alberta
in partial fulfilment of the
requirements for the degree of
MASTER OF SCIENCE

Edmonton, Alberta
September, 1948

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J. D. Gilpatrick

INTRODUCTION

Ophiobolus graminis Sacc., the cause of Take-all of wheat and other members of the Gramineae is a destructive parasite in many of the wheat-growing areas of the world. Although several aspects of this fungus have been carefully studied its fruiting habits until recently have been given little more than passing attention in the literature. Since this phase of the organism's activity has been neglected, the studies reported here were undertaken.

O. graminis fruits by the production of but one spore stage. Fruiting bodies called perithecia bearing the spores, when formed on the host, are found near the soil level. These bodies however are not always formed in nature and are rarely found on infected wheat plants in Alberta fields.

Their occurrence in this province is apparently much less common than in certain other wheat-growing regions such as those of the eastern United States and the British Isles. That sporulation occurs only rarely in pure culture has been reported by Davis (16), Davies (14), Russell (72), Hynes (40), and Garrett (25) and has been confirmed by observations made over several years at this laboratory.

A better knowledge of the factors affecting sporulation of O. graminis would be useful for several reasons. Firstly, any method of inducing the organism to fruit on diseased tissue would facilitate isolation which is ordinarily difficult if ripe perithecia are not present. Secondly, isolates could be more readily and conclusively indentified. Thirdly, a sufficient supply of spores would be available for genetical studies. Fourthly, O. graminis exists as many different strains in nature and several of these may be isolated from a single area. Probably these strains differing in pathogenic capabilities on the host and cultural characters on agar arise through the sexual process. Thus a simple method of inducing fruiting is necessary for studies of physiologic specialization of this organism. Fifthly, although Garrett (23) has concluded that it is very unlikely that spores can play any part in the dispersal and survival of this organism under field conditions, there seems to be need for further studies on the relationship of ascospore

production to the epiphytology of "Take-all". A plentiful supply of spores produced at will would be required for such studies. Finally, a knowledge of the factors promoting fruiting of this fungus, which sporulates erratically, would probably contribute to the understanding of the sporulation of fungi in general.

The purpose of the work reported here was to contribute to the understanding of the mechanism of sporulation by studying in pure culture and on the host the effect of various factors on this process. Furthermore, attempts were made to develop a simple repeatable technique for the production of mature perithecia.

Several factors have been suggested as playing a part in the fruiting process of O. graminis. None as yet has been demonstrated as being critical. Physical, nutritional, and biological factors affect the sporulation of fungi in general. Of the physical factors, light, soil moisture, and humidity are important. Nutritional aspects include both the quality and quantity of food. The interaction of associated microorganisms with O. graminis may be an important biological factor.

Biological and nutritional factors are stressed in the present study. Although physical conditions receive less direct attention they are always kept in view when other factors are under consideration and are maintained at levels thought likely to be optimum for the sporulation of the organism.

DESCRIPTION OF OPHIOBOLUS GRAMINIS

The life cycle of many fungi consists typically of a mycelial or vegetative stage and two reproductive stages - sexual and asexual. Although conidial or asexual spore stages of O. graminis have been reported, their occurrence has never been confirmed. The sexual stage is characterized by the production of flask-shaped perithecia in which are borne banana-shaped sacs or asci, each containing 8 ascospores. The spores are long, slender, hyaline, septate, and lie parallel in the ascus. These structures are illustrated in Figures 1 and 2.

Measurements given by various workers for the length of ascospores of this organism vary from 60 to 118 microns (72). Ascospore lengths were measured from time to time in the present study. In one instance spores from perithecia formed on wheat plants, grown under ordinary conditions in the greenhouse, varied from 60 - 111 microns in length. The mean length of 100 spores was 92.9 ± 19.2. These ascospore lengths seem to agree with those previously reported in the literature.

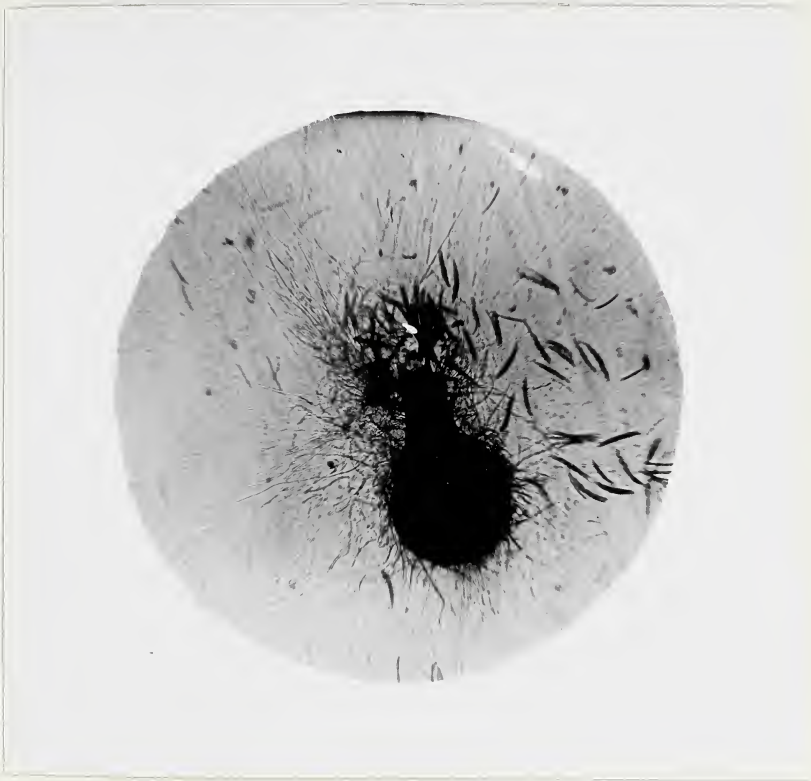

Figure 1

Perithecium and asci of Ophiobolus graminis
Sacc. formed in pure culture -
after F. R. Davies (14)

Figure 2

Asci and ascospores of Ophiobolus graminis from a perithecium formed on a wheat seedling. Note that the septate ascospores lie parallel in the ascus.

Figure 3

Wheat seedling infected with Ophiobolus graminis by the flask method. Note the unusually long, protruding beaks of the perithecia of the organism curving upward toward the light.

O. graminis attacks the basal parts of the host from the roots to several inches above the crown. A plate of brown mycelium forms between the outer and inner leaf sheaths and between the inner leaf sheath and the culm. It is from these plates of mycelia that the perithecia arise. When they are mature their curved beaks may sometimes be seen protruding through the outer leaf sheath (Figure 3).

In the class Ascomycetes, of which O. graminis is a member, the development of asci and perithecia seem to be two separate processes, the ascus resulting from a sexual process and the perithecium arising from vegetative hyphae. Both processes might be dependent on the same stimulatory effect or different effects. If there are different mechanisms then conceivably a perithecium or ascus might be induced to form without the other structure being present. Although naked asci have never been reported, perithecia-like bodies which bear no asci are often observed in artificial culture. These structures are usually regarded as immature perithecia.

Jones (41) has described cytological details of the development of mature perithecia of O. graminis*. He found that ascospore production is initiated by the conjugation of two or more vegetative cells. From such conjugated cells ascogenous hyphae arise with binucleate cells. This process occurs within a fairly well developed perithecium. Ascus development appears to take place from any binucleate cell of an ascogenous hyphae. Nuclear fusion occurs in the young ascus. This is followed by three divisions of the nuclei one of which is a reduction division. The resulting 8 nuclei are then walled off one to a unit and each unit eventually develops into an ascospore.

Thus according to Jones' description the initiation of perithecial formation is not dependent on the conjugation process. Therefore it is possible to conceive stimulation of perithecia without further stimulation of ascus formation. In the light of available information this conception is purely speculative.

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* Since Jones' work was reported, Miss Turner (Trans. Brit. Mycol. Soc. 24:269-281. 1940) has demonstrated that certain isolates of O. graminis from oats are distinctly different from those of wheat and she regards them as belonging to a new variety avenae. It is possible that the strains that Jones worked with belonged to this variety.

In these studies two isolates of the organism designated as S_1 and S_2 were characterized by the formation of small, black spermagonia-like bodies in the presence of certain growth factors. These were usually covered with a tuft of white mycelium. When formed in the centre of a culture they were roundish in shape (Figure 4), but when formed at the junction of glass and agar they were ovoid and possessed long, beak-like appendages. These bodies produced no asci or ascospores but at times were observed to exude tiny structures resembling spores the exact nature of which was not determined. Several strains including S_I formed similar bodies which were naked and never covered with a tuft of mycelium. Nor were they ever observed to exude spore-like bodies.

Jones (41) reported that O. graminis forms spermagonia and spermatia (male sex elements) but he considered them to be functionless. At maturity the spermagonia were spherical or ovoid in shape. They produced small cells of narrow diameter, blunt at one end and narrowing to a point at the other extremity. These bodies measured from 5 - 7 microns by 1 - 1.5 microns, were distinctly curved, and possessed a prominent nucleus. These same structures have not been reported by other workers.

The small spore-like bodies observed in these studies may have been of the same nature as those observed by Jones. However they were much shorter, possessed no prominent nucleus, and did not appear constricted at one end. However, some of them were definitely curved. The black-bodies produced in these tests which bore tiny spores possessed long beaks or appendages under certain conditions. Jones' spermagonia were not so characterized. Because of the nature of the spores produced by these bodies in these studies they are not perithecia. However it seems logical to assume that they may be spermagonia similar to those produced by other members of the Ascomycetes closely related to O. graminis.

It is possible that the non-spore-forming black bodies produced in these tests were immature perithecia. The proper conditions may not have been provided for conjugation to take place or for the subsequent development of mature ascospores.

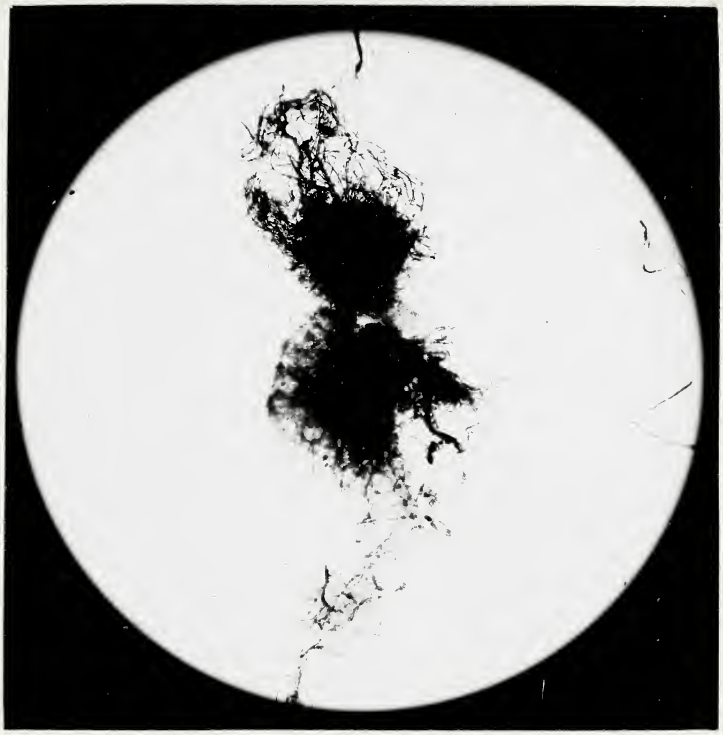

Figure 4

Spermagonia-like bodies produced by strains S_1 and S_2 of Ophiobolus graminis in pure culture.

SEXUALITY OF *O. GRAMINIS*

Introduction

Fungi which require the intermingling of thalli of different strains before sexual reproductive bodies will form are spoken of as being heterothallic. Those fungi requiring no such interaction of strains are described as homothallic.

O. graminis is known to have many different strains with varying pathogenic capabilities on the host and different morphological characteristics in pure culture. This physiologic specialization has been observed at this laboratory and by workers elsewhere (25, 60). Russell (73) has discussed the subject in a separate paper.

Because *O. graminis* exists as different strains the question arises as to whether the organism is homo- or heterothallic, and as to whether different strains differ in their sporulating abilities. These problems were investigated in the following studies in an attempt to determine the role that physiologic specialization may play in the sporulation of *O. graminis*.

Studies with Mixed Strains

Literature Review

The sexuality of O. graminis has been investigated by several workers. Kirby (46) reported that all of his New York strains of this organism were heterothallic. He stated that in order to get sporulation in pure culture it was necessary to grow + and - strains together. This same worker, however, later found other isolates which would produce perithecia without the stimulus of a second strain (47).

Davis (16) found no evidence of heterothallism when a single spore isolate originating from one of Kirby's "heterothallic" New York strains was grown in agar culture. This isolate sporulated as well when grown alone as it did when in combination with two other strains. The latter failed to sporulate under any conditions of the environment. Davis felt that this lack of vigor was the result of long culture in an artificial medium. He noticed an interaction along the line where two colonies of the same or of different strains approached each other. This was characterized by a darkening of the mycelium of all strains and the formation of perithecia by the New York strain.

Davis concluded that although his New York strain was homothallic that nutrition or the products of metabolism may act as a stimulant for sporulation whereas otherwise the organism would not fruit. He suggested that this postulation might explain Kirby's results.

Both Davies (14) and White (84) found evidence in favor of homothallism but could offer no support to the theory of a nutritive stimulus. Davies reported that monosporous cultures of the organism produced perithecia abundantly in Petri plates under certain conditions. Growth of four different strains in combination did not induce fruiting in one case although two of the strains grown separately on diseased wheat seedlings produced perithecia. White found that monosporous isolates of O. graminis formed perithecia when grown on roots of wheat plants in pure culture on nutrient agar in large tubes. He also found that 8 monosporous isolates grown on potato dextrose agar sporulated no more readily in combination than alone.

Experimental Studies

With evidence for and against a nutritive stimulus for reproduction and with the absence of a satisfactory explanation of Kirby's results, apparently there is a need for further investigations of this problem. Consequently

two experiments involving mixed strains were conducted in these studies: one on agar in Petri plates; the other on wheat seedlings in pots.

1. Petri plate studies

Various strains of O. graminis were grown singly and in various combinations on potato dextrose agar in Petri plates which were incubated at 15° - 20°C in diffuse natural light conditions in the greenhouse. Bits of mycelium of the strains were transferred from agar slants to the surface of the medium in the plate. Two plantings spaced about 50 mms. apart were made in each plate. The cultures were observed for 8 weeks before discarding. The strains used and the results of the experiment are presented in Table 1.

No perithecia were formed in any of the plates. The rough type of growth produced by strains S_1 and S_2 is characteristic of these strains on various media. (See Figure 5). The black bodies resembling spermagonia were formed over the plates but most abundantly where two colonies approached each other or at the point of contact of agar and glass.

TABLE I

COLONY INTERACTION OF DIFFERENT STRAINS OF *O. GRAMINIS*

Strains in combination	Number of perithecia produced	Antagonism between colonies
8; 8	0	0
8; III-2	0	++
8; III-14	0	++
8; III-17	0	+
8; S_1	0	++
8; S_2	0*	+
S_1; S_1	0*	+
S_1; III-2	0*	++
S_1; S_2	0*	++
S_2; S_2	0*	+
III-2; III-2	0	0
III-2; S_2	0*	++

* = Black tufts resembling immature spermagonia produced by strains S_1 and S_2.

0 = No perithecia or no antagonism.

\+ = Slight antagonism.

++ = Moderate antagonism with definite clean zone between colonies.

Antagonistic reactions occurred where two colonies of different strains approached each other; between colonies of the same strain there was only slight or no inhibition (See Figure 5). A ruffled type of growth was observed along the line of approach of antagonistic colonies.

2. Pot studies

In a similar test carried out in seven inch pots in the greenhouse, highly pathogenic strain 8 was used singly and in combination with 4 other less pathogenic strains as inoculum for infecting wheat. These strains were grown separately on a soil medium containing 10% corn meal by weight. The single strain inocula thus produced were added to the pots of sterilized soil at seed level. When inocula of two different strains were used together, equal amounts of each were added. The total amount of inoculum added to each pot was 75 gms. Twenty-five seeds of Red Bobs wheat were sown in each pot and covered with one inch of sterile soil. The plants were harvested and examined for perithecia 8 weeks after emergence. The results are presented in Table II.

TABLE II

EFFECT OF MIXED INOCULUM ON PERITHECIAL PRODUCTION AND PATHOGENICITY

Strains used as inoculum	Perithecial production	Pathogenicity rating
8	0	5
8;III-2	0	3
8; IV	0	3
8; S_1	0	3
8; S_2	0	3
Check (No inoculum)	0	0

Although infection was fair in all pots, no perithecia were observed and the pathogenicity of the mixed cultures was reduced over that of strain 8 alone.

Discussion

Colonies of mixed strains were moderately antagonistic in pure culture in these studies. This phenomenon was not exhibited by colonies of the same strain. Although this strain interaction exhibited was not characterized by

perithecial production as reported by Kirby, the appearance of a ruffled type of growth is interesting. It is unlikely that this phenomenon was due to a competition for nutrients since it was not observed to occur between colonies of the same strain. The interaction suggests some nutritive, hormonal or antibiotic activity between strains. Such an explanation might account for Kirby's conclusions that there are + and - strains of this organism.

In the pot studies, there was no interaction between different strains which stimulated perithecial formation on the host. The reduction of the pathogenicity of the mixed cultures over that of strain 8 alone might suggest some strain interaction. However these four strains are all less pathogenic than strain 8. Thus it is likely that the reduction of pathogenicity is due to a dilution effect similar to that observed by Henry and Gilpatrick (37) when working on the relative pathogenicity of single and mixed strains of this organism.

Studies with Different Strains

It seems likely that certain strains of this organism may be more fertile than others. Davis (16) observed that one of his strains would fruit readily in pure

culture whereas two other strains were never observed to produce perithecia under the same conditions. Russell (72) and Davies (14) also observed that some strains produced perithecia more readily than others.

Many other fungi are composed of strains with different sporulating abilities. Leonian (51) in a study of Valsa leucostoma found this fungus to possess two strains: one capable of giving rise to both the perfect and imperfect stage; the other incapable of developing perithecia regardless of the environment. Different abilities of different strains to carry out asexual reproduction in pure culture have been observed by Hansen (28) for Botrytis, by Keitt and Langford (43) for Venturia inaequalis, and by Kernkamp (45) for Ustilago zeae. Westergaard and Mitchell (83) developed a synthetic medium for perithecial formation by certain wild type crosses of Neurospora which was unsatisfactory for the fruiting of certain mutants with specific growth requirements.

The possibility also exists that O. graminis may lose its fertility in pure culture, particularly since this organism may lose its pathogenicity under the same conditions as has been observed by workers at this laboratory and by Russell (72) and since other fungi lose their fertility after a period in artificial culture.

Ryker (74) reported that cultures of certain species of Cercospora ultimately produce feebly sporulating variants and that this was due to the suppression of the original cultures by non-conidial variants. Chilton (11) has reported a similar situation for Colletotrichum destructor. During an investigation of the physiology of perithecial production in Melanospora destruens, Hawker (32) found that the strain in use became progressively less fertile. This investigator observed that during subculturing at laboratory temperature growth of sterile saltants began earlier than that of fertile strains. However at higher temperatures this difference was greatly reduced.

Experimental Studies

The ability of different strains of O. graminis to sporulate was investigated as well as the possibility of the loss of fertility in artificial culture.

Over a period of two years, 18 different strains were never observed to sporulate on potato dextrose agar in pure culture. At times other media were also used. Cultures were always incubated at about 20°C in the greenhouse or near a north window in the laboratory. Ten of

the isolates had been cultured for almost 20 years. The other 8 cultures were freshly isolated.

In one experiment, 5 recent isolates were grown singly on agar media while strains S_1[1] served as a check. The two media used were wheat-stem agar and a basic nutrient medium[2] to which biotin and thiamin were added at the rate of 100 μg. and 1,000 μg. per litre respectively. The strains used and the relative number of perithecia-like black bodies produced are recorded in Table III.

TABLE III

RELATIVE ABILITY OF DIFFERENT STRAINS TO PRODUCE PERITHECIA-LIKE BLACK BODIES ON VITAMIN AND WHEAT STRAW AGARS

Strain[3]	Vitamin agar	Wheat-stem agar
A45	None	Few
A47	Few	None
A49	None	None
A50	Few	None
A60	Many	Few
S_1	Many	Few

1 = Isolated by S. Goto at this laboratory in 1943.

2 = Basic medium described on Page 82.

3 = The writer wishes to express his thanks to Dr. L. E. Tyner, Dominion Laboratory of Plant Pathology, Edmonton for kindly supplying the recent isolates used in these studies.

The tendency toward the production of the perithecia-like bodies on the media tested is apparently greatest with strain A60 and S_1. It is also interesting to note that these bodies were produced more abundantly on the vitamin agar than on the wheat-stem agar.

In another experiment, the relative ability of 6 isolates to sporulate on the host was investigated. Three strains - A49, A60, and A71 were recent isolates. The other strains had been in culture for 4 - 17 years.

Wheat plants were infected according to the flask method as described in the section on physical factors. In this method wheat seedlings are planted asepticly in small Erlenmeyer flasks containing the fungus growing on a mixture of soil and corn meal. In this experiment no attempt was made to maintain aseptic conditions after good infection was apparent and the bungs of the flasks were removed at that time exposing the cultures to contamination from the air.

After 8 weeks, the plants in the flask were examined for perithecia. The relative pathogenicity and perithecial production are presented in Table IV.

Under the conditions of this experiment, strains A71, A49, A60, and 8 were highly pathogenic, killing the host plant outright in most cases. However, strains III-17 and S_1

were only slightly pathogenic which probably accounts for their inability to form perithecia. Strain S_1 has on the other occasions produced perithecia on the host when exhibiting moderate pathogenicity. Among the pathogenic strains, A60 was almost sterile, strain 8 moderately fertile, and strains A49 and A71 produced perithecia abundantly.

TABLE IV

RELATIVE PATHOGENICITY AND FERTILITY OF DIFFERENT STRAINS ON THE HOST

Strain	Replicates a	b	c	d	e	f
A49	5 ++++	5 ++++	5 ++++	5 ++++	5 ++++	5 ++++
A60	5 -	5 +	5 -	5 -	5 -	5 -
A71	5 ++++	5 ++++	5 ++++	5 ++++	5 ++++	5 ++++
8*	5 +++	5 ++	3 -	5 ++	5 ++	
III-17	0 -	2 -	0 -	2 -	2 -	0 -
S_1	1 -	2 -	2 -	2 -		

* = Strains 8 and III-17 isolated by F. R. Davies at this laboratory in 1931.

For meanings of figures and symbols see next page.

Numerical figures represent pathogenicity rating.

0 = Non-pathogenic
1 = Trace of pathogenicity
2 = Slight pathogenicity
3 = Moderate pathogenicity
4 = Severe pathogenicity
5 = Plants killed

Algebraic signs represent relative perithecial production.

\- = No perithecia
\+ = Few spores observed when tissue was examined
++ = Few perithecia per plant (1 - 5)
+++ = Several perithecia per plant (6 - 10)
++++ = Many perithecia per plant (more than 10)

Discussion

From the results of observations made on the relative ability of different strains to sporulate, it appears that some strains are more fertile than others under the same environmental conditions. These results also indicate that the culturing of strain 8 for 17 years has not caused this strain to become sterile. Furthermore, strain S_1, an isolate of many years, has produced perithecia

on other occasions in these studies. Thus with these two strains at least, fertility has not been completely lost by their long culturing on an artificial medium.

The inability of strains A49, A71, and 8 to sporulate in pure culture is evidently not due to their infertility but more probably the result of improper nutritional or physical conditions. Favorable conditions are evidently provided by the host plant under the environment present in the experiment described.

The fact that single strains sporulated on the host in the present studies supports the observations of Davis (16), Davies (14), and White (84), that *O. graminis* is homothallic.

EFFECT OF PHYSICAL FACTORS ON SPORULATION OF FUNGI

Introduction

The physical elements of the environment such as light, humidity, and temperature seem to play their part in the reproductive life of the fungi. Many fungi sporulate well in nature where the physical conditions are evidently satisfied. However, in pure culture some fungi do not sporulate readily and it is possible that under these conditions some physical factor is not satisfactorily provided. The following literature review although incomplete illustrates the importance of light, temperature, and humidity on the sporulation of a few fungi.

Literature Review

Light

Probably the most extensively studied physical factor affecting the fruiting of fungi is light. Many workers have reported that light helps to initiate, increase,

and hasten sporulation. Ramsey and Bailey (65) found that a strain of Fusarium coeruleum which had never produced conidia in culture did so following exposure to ultra violet radiations. These same workers found that spore production of Macrosporium tomato and Fusarium cepae was increased greatly by exposure to the same radiations. Hutchinson and Ashton (39) obtained numerous acervuli and conidia of Colletotricum phomoides within 24 hours after radiating by means of a mercury arc lamp. Under normal conditions acervuli do not usually develop until the 8th day.

Light may modify spore characteristics. The conidia of three Fusarium species studied by Harter (29) reached their greatest length in cultures exposed to a maximum amount of daylight while those produced in the dark were shorter.

Dillon-Weston (19) demonstrated that the quantity of light may be of importance. With the several species studied he found that long exposures to ultra violet inhibited whereas short exposures stimulated sporulation. He also noted that under ordinary laboratory conditions the light intensity is not great enough to induce Helminthosporium avenae or Altenaria solani to sporulate. Snyder and Hansen (80) reported that Centrospora acerina sporulated profusely

on agar media outside a north window but not at all inside the window. The same is true of the imperfect stage of Botryosphaeria ribis and Mycosphaerella brassicola. Houston and Oswald (38) studied the influence of photoperiodism on the sporulation of Helminthosporium gramineum. They found that exposure to light - preferably daylight - is necessary for conidial development on agar.

Temperature

In a recent review of the literature on the effect of temperature on the reproduction of fungi, Wolf and Wolf (87) concluded that there is abundant evidence that temperatures favorable for the growth of fungi may be slightly lower than those for reproduction. They cite many illustrations of this relationship. However, Cherewick (10) found that chilling of plants infected with Erysiphe graminis increased perithecial formation and observed that alternating temperatures brought about greater sporulation.

Keitt and Langford (43) demonstrated that temperature greatly influences perithecial formation of Venturia inaequalis. Mature perithecia were produced abundantly in pure culture in about 5 months if incubated at 8°C.

Fruiting was still abundant but took longer at 4°C. An abundance of initials but only an occasional mature perithecium with ascospores was found in cultures incubated at 12°C, while only small initials and no ascospores were produced at 16°, 20°, and 24°C, respectively. Further tests showed that mature perithecia could be obtained in a shorter time by incubating the plates at about 20°C for the first 10 days to produce rapid vegetative development then lowering the temperature to 8°C until the early ascus stage was reached and then raising the temperature to about 15°C. If however the temperature was raised before the perithecia were sufficiently advanced, no ascospores were formed.

Humidity

This factor seems to play an important part in the development and maturation of fruiting bodies. Sporangia of Phytophthora infestans form readily only in saturated atmospheres. No sporangia are formed by this organism at a relative humidity less than 91 percent (13). Alternate drying and wetting of perithecia is necessary to induce the formation of ascospores by Erysiphe graminis but other stages of the sexual process are favored by relatively dry conditions (10). Dixon et al. (20) observed that

sporulation of the tobacco downy mildew fungus (Peronospora hyoscyami) is favored by long periods of saturated humidity at a time when skies are overcast. Longree (55) demonstrated that decreasing the relative humidity gave sparser mycelial development and fewer conidiophores with Sphaerotheca pannosa var. rosae on young rose leaves, but that both events occurred even at 21 - 22% relative humidity.

Physical Factors and the Sporulation of O. graminis

Literature

There is little available information on the effect of physical factors on the sporulation of O. graminis. Davis (16) was unable to find perithecia in cultures of this organism incubated in the darkness but often observed them in cultures held in the daylight. However, he points out that occasionally perithecia are found on the roots of plants near the crown where they are formed in the darkness. Davies (14) reported that 4 strains which failed to sporulate in darkness, produced perithecia when transferred to the daylight of the laboratory. This author also studied the effect of ultra violet light but found that exposure from 2 to 15 seconds did not stimulate perithecial production to any great extent. Both Russell (72) and Garrett (25)

consider that light exerts a favorable influence on sporulation.

Russell (72) reported that exposures of cultures to low temperatures seemed to stimulate the production of perithecia. Davies (14) incubated cultures at different temperatures for 4 weeks and then transferred these to the laboratory where they were placed at room temperature in the light. No significant effect of temperature on fruiting was observed.

Moisture and humidity probably influence perithecial formation of O. graminis. As previously pointed out, perithecia are commonly found on diseased wheat plants in the British Isles and in the eastern United States where the relative humidity and rainfall are usually much greater than in Alberta where perithecia are less commonly observed. In New South Wales, Hynes (40) considers that the development of perithecia is dependent on adequate moisture supplies.

Experimental Studies

Methods of Inducing Sporulation

Techniques for inducing sporulation of O. graminis have been devised taking into consideration the effect of

physical and other factors. A few of these methods have been investigated in these studies to ascertain their relative value.

Hynes' Method

In this method, Hynes (40) moistened the butts of wheat plants affected with Take-all collected from the field, and placed them in Erlenmeyer flasks under laboratory conditions. Fruitifications soon developed.

In testing this method, severely infected wheat seedlings were harvested from artificially inoculated soil in pots in the greenhouse. These plants were washed and placed in 250 cc. flasks with 10 seedlings per flask. A small amount of water was added to each container. Three flasks were then incubated under each of the following conditions:

(1) In a humidity chamber in the greenhouse in diffuse sunlight at 15° - 20°C.

(2) In the greenhouse in bright sunlight at 20° - 30°C.

(3) In a north window in the laboratory at 18° - 22°C.

(4) In a temperature control chamber in the laboratory at 18°C in the dark.

Moisture was added to these flasks as required.

At the end of 8 weeks these plants were examined but no perithecia were observed.

Garrett's Method

Perithecia were obtained in abundance by Garrett (23) when using the following method:
wheat seedlings were planted above agar inoculum of the fungus in large tubes containing sand moistened with a standard nutrient solution for wheat. The tubes were incubated in glass jars at 16° - 20°C in a north window. No attempt was made to maintain sterile conditions. Mature perithecia were obtained in about 6 weeks.

This method was tested on two occasions in the present studies, but unfortunately in both cases only poor infection resulted, so that the method could not be appraised.

White's Method

White (84) obtained favorable conditions for perithecial production by inoculating the roots of wheat plants in pure culture on nutrient agar in large tubes. Four weeks later the plants died and cultures were left to dry out. Mature perithecia developed in about 8 - 10 weeks and formed along the roots at the junction of the agar and glass.

To test this method, sterile wheat seedlings were added to potato dextrose agar slants upon which O. graminis was growing. The slants were incubated in diffuse light in the greenhouse. Although good infection resulted, no fruiting bodies appeared.

Possibly better success might have been achieved with this method if it had been tested further with the use of other media.

Flask Method

A successful method for producing perithecia on the host plant was devised during the present study. This consisted of growing wheat plants in small flasks containing artificially inoculated soil. Fifty grams of a mixture of soil and corn meal (10 percent by weight) was added to small Erlenmeyer flasks and moistened with 32 ccs. of distilled water. The flasks were bunged and sterilized in the autoclave at 15 pounds pressure for two hours. A small bit of O. graminis mycelium growing on an agar slant was used as inoculum. After a 21-day incubation period, the flasks were planted aseptically with 5 Red Bobs wheat seeds which had been previously surface sterilized with 1:1000 mercuric chloride and rinsed with sterile water. At this same time 5 ccs. of water was added to each container to provide adequate moisture for the growth of the host.

These planted flasks were incubated in a humidity chamber in the greenhouse at from 15° - 20°C. Direct sunlight was never allowed to fall on the plants but light intensity during the day was high. Usually moisture was condensed on the inside of the flasks at least to a height of 20 cms. above the soil line.

Good infection was apparent 15 days after seeding. The plants were dead and perithecia could be observed at the end of 6 weeks.

Sterile conditions were maintained in about 50 percent of the flasks. A Helminthosporium species which was introduced through the seed was the most common contaminant. On some occasions the bungs were removed from the flasks after sowing the seed in order to allow recontamination of the soil. Infection was not appreciably reduced by recontamination when highly pathogenic strains were used.

Discussion

Of the four methods tested in these studies, the flask method was the only one which was used successfully for the production of perithecia. The success of this method was probably due in considerable measure to the fact that the substrate for the organism was the host plant, that good

infection was obtained, that the relative humidity was high, and that abundant daylight was provided. Garrett (23) concluded that these same conditions contributed to the success of his method. He also considered that the maintenance of non-sterile conditions was important. Possibly the non-sterile conditions often prevailing when the flask method is used contribute to its success. This point will be more fully discussed in the section dealing with the microorganismal factor.

Each of the methods described involved the use of the living host plant as the substrate. Such methods have definite limitations. A general lack of uniformity exists resulting from biological variations among different plants. It is difficult to establish uniform infection in each plant. Furthermore, any environmental factors acting on the organism are also acting on the substrate or host plant. Thus the substrate is not likely to be uniform under different conditions of environment. A method making use of a substrate that could be duplicated would be preferable.

The Effect of Light and Diurnal Effects on Sporulation

An attempt was made to determine the importance of outdoor environment on the sporulation of *O. graminis*.

Ten slants each of 3 percent malt agar and potato dextrose agar were inoculated with strain S_1 and placed outdoors in contact with the soil and exposed to daylight. Ten inoculated slants of each medium were also retained in the laboratory at room temperature. Rather cool conditions, 5° - 25°C, prevailed outdoors during the experiment.

No perithecia were observed after 60 days in either the outdoor or indoor cultures. Very rough growth occurred on malt agar under both conditions and numerous black, perithecia-like bodies were formed.

The outdoor cultures were left out for another 4 weeks during which time freezing conditions occurred on a few nights. These cultures were then returned to the laboratory and incubated at room temperature in a north window. After two weeks they had dried out. At this time they were revived by adding water and at the end of 10 days were examined for perithecia but none were found.

Evidently the conditions of the laboratory or the field did not provide a satisfactory environment for perithecial formation by this strain on the media tested.

The effect of photoperiodism on sporulation was also studied. Wheat plants were infected with the organism using the flask method and maintaining as sterile

conditions as possible. After the plants had become severely infected and were starting to die, six flasks each containing 5 plants were placed under each of the following light environments with temperature and humidity held constant:

(1) continuous darkness

(2) 4-hour day

(3) 8-hour day

(4) 12-hour day

At the end of 4 weeks, the plants were examined for perithecia and the lengths of ascospores under each light environment were measured. The results are presented in Table V.

TABLE V

THE EFFECT OF LIGHT DAYS OF DIFFERENT LENGTH ON RELATIVE PERITHECIAL AND ASCOSPORE PRODUCTION AND ASCOSPORE LENGTH

Treatment	Number perithecia	Number ascospores	Ascospore length in microns	
			Range	Mean length (100 spores)
Continuous darkness	++++	+	56-79	67.9 ± 3.4
4-hour day	++++	++++	60-80	68.5 ± 3.1
8-hour day	++++	++++	65-78	64.5 ± 3.7
12-hour day	++++	++++	62-80	70.5 ± 3.6

Length of day had little effect on the relative number of perithecia or ascospores produced. Furthermore there were as many perithecia produced in darkness as in the light. However few perithecia formed in the dark bore mature ascospores. The length of day had no marked effect on ascospore length. However it is interesting to note that the mean spore lengths under these conditions were much shorter than for those formed in a normal day (See page 5).

Light evidently stimulates the maturation of perithecia and the production of ascospores by this fungus. In this experiment, possibly perithecia had started to form at the time the plants were placed in the various light environments or possibly the host or fungus had received some stimulus which induced perithecial formation later. Further exposure to light was probably necessary before these perithecia could produce an abundant supply of ascospores.

Light had a marked effect on the length and direction of growth of the beaks of the perithecia. In the dark, beaks were shorter and undeveloped; those in the light were long and slender and often bent upward toward the light. This phototropic effect is illustrated in Figure 3.

The Effect of Other Physical Factors on Sporulation

Temperature

Although a few experiments were conducted on the effect of temperature on sporulation, results were always unsatisfactory and not worthy of recording here. However, it was generally observed that perithecia were formed at relatively cool temperatures (15°C). This agrees with Russell's suggestion (72) that sporulation of *O. graminis* is favored by cool conditions.

The optimum growing temperature for strain 8 ranged from 15° - 25°C. Fair growth occurred at 10°C and very poor growth at 30° and 35°C.

Humidity

Though no special humidity experiments were made, it was noted that perithecia were formed only when the humidity was high and when the host or soil was not allowed to dry out.

Soil Moisture

An experiment was conducted to test the effect of soil moisture on sporulation. Wheat seedlings were infected by sowing seed in pots containing soil artificially inoculated with 5 different strains of O. graminis. Early in July, after infection had occurred, the seedlings were planted outside in natural soil in two lots and fully exposed to the elements. One lot was watered heavily every 2 or 3 days whereas the other was left unwatered. For the first 3 weeks in the field, conditions were very dry and there was a great difference in the soil moisture content of the watered and unwatered plots. However a wet season followed and continued into September. At the end of this month all plants were harvested and examined for perithecia but none were found even though many plants were severely lesioned.

It is interesting to note that perithecia did not form on any of the diseased plants although in the case of one lot, the moisture content of the soil was high throughout the entire period.

Discussion

From the results of these experiments and from the reports in the literature it appears that physical factors play a large part in the sporulation of *O. graminis*. The role of light appears to be quite significant. Light was shown to be necessary for the maturation of perithecia and to have a marked effect on the morphological characters of the perithecia.

In the experiments reported, perithecia were only produced on the host when abundant light, high humidity, and fairly cool temperatures were provided. When studying other factors, these physical conditions were maintained as closely as possible to these levels in an attempt to supply favorable physical conditions for sporulation.

The failure of 5 strains of the *O. graminis* to sporulate on plants growing in soil with a high moisture content suggests that this factor is not the only one which governs sporulation of this fungus.

THE EFFECT OF MICROORGANISMAL FACTORS ON SPORULATION

Literature Review

It is a well established fact that many fungi sporulate more readily or more profusely when in association with other microorganisms in artificial culture than when grown alone. Asthana and Hawker (2) have reviewed the literature dealing with this subject up until 1936. They also report that sporulation of Melanospora destruens and some other Ascomycetes was stimulated by the presence in the culture plates of certain organisms or products derived from the latter. Gwynne-Vaughan (27) in a review of the question of sex and nutrition of the fungi concluded that heterothallism in Humaria granulata apparently is not based on a difference in sex but on a difference in metabolism of the one strain brought about by the presence of the second. Nickerson and Thimann (59) showed that Aspergillus niger produces a substance which promotes conjugation of three Zygosaccharomyces spp. which conjugate only sparingly

in pure culture. Dead cells of these Zygosaccharomyces contain some of this substance which probably induces some conjugation.

Hazen (36) has recently demonstrated that Microsporium audouini is deficient in certain factors essential for its profuse growth and for the development of macroconidia. These factors may be produced by a bacterium, Bacillus weidmaniensis.

Sporulation of O. graminis is also influenced by other organisms with which it associates. In his Master's thesis presented to the University of Alberta in 1932, Davies (14) reported that the presence of an unidentified bacterium stimulated the abundant production of perithecia by O. graminis in artificial culture. He found that only the living bacterium had this stimulatory effect. Sterile extracts from liquid media in which the bacteria had grown did not induce perithecial formation. The foregoing and other findings on this subject made at this laboratory have been reported recently by Davies et al. (15). In one experiment 96 percent of wheat seedlings grown in unsterilized artificially inoculated soil produced perithecia, whereas only 8 percent of the seedlings in sterilized soil produced them. These same workers concluded that "the

ability to stimulate sporulation does not appear to be possessed by many individual microorganisms since a survey of more than 450 isolates of fungi, bacteria, and actinomycetes from the soil, the basal parts of wheat plants, and other sources failed to disclose this property in any of them".

Reviews of the extensive literature dealing with the effect of other microorganisms on growth, pathogenicity, and survival of O. graminis are presented by Garrett (25) and Slagg and Fellows (78).

Broadfoot (7) studied the growth relationships of 66 cultures of bacteria and fungi toward O. graminis on agar media and on the host. He found that any growth reaction on the two solid media used was not a reliable indication of what happens on the host and that different growth relationships occur on different media. The suggestion was made that various microorganisms, each with its complement of enzymes, might produce substances which differ in kind and amount according to the substrate.

Slagg and Fellows (78) found that some fungi produce substances that inhibit the growth of O. graminis in pure culture, whereas the by-products of other fungi act in a stimulatory manner. They observed that the

production of these products by any fungus varied with its stage of growth and with the nature of the substrate. They also noted that some fungi may produce inhibitory by-products on one culture medium and stimulatory by-products on another. Neither these workers nor Broadfoot reported on the effect of other microorganisms on the sporulation of *O. graminis*.

Experimental Studies

The following experiments were conducted in an attempt to obtain further information on the effect of various microorganisms on the growth and sporulation of *O. graminis*.

Experiment I

The effect of 98 different isolates on the sporulation of *O. graminis* was studied on potato dextrose agar in Petri plates. Seventy-three of these organisms were isolated from the soil around wheat stubble gathered

from the field. The isolates included 11 fungi, 24 actinomycetes, and 38 bacteria. The remaining 25 organisms used were available in the laboratory as named isolates and included the following:

Bacteria

Bacillus megatherium
B. mycoides
B. mesentericus
B. niger
B. brevis
B. cereus
B. vulgatus
B. subtilis - strain A16
B. " - " A30
B. " - " A31
B. " - " A32
B. " - " A33
B. " - " A231

Aerobacter aerogenes
Pseudomonas atrofaciens
Pseudomonas coronafaciens
Erwinia amylovora
Erwinia carotovora
Phytomonas insidiosa
Phytomonas tumefaciens
Corynebacterium sepedonicum

Fungi

Sclerotinia sp.
Penicillium sp.
Cladosporium sp.

The inoculum of the above organisms was introduced onto the agar medium about 30 mms. distant from a week old colony of *O. graminis* (strain 8). The plates were incubated in the

greenhouse in a humidity chamber. They were examined at the end of 8 weeks. No perithecia were observed.

The inability of the 98 organisms tested to induce O. graminis to sporulate in agar culture seems to support the suggestion of Davies et al (15) that very few organisms have this ability.

Experiment 2

1. Plate Studies

A study was made of the effect of time of inoculation on the interaction between O. graminis and other organisms in agar culture. Petri plates containing 25 ccs. of potato dextrose agar were planted with a bit of culture of strain 8. One series of these plates was also planted with 8 other organisms (one per plate). All plantings were about 30 mms. apart. A second series was planted when the O. graminis cultures were 5 days old; a third at 10 days. Trichoderma was added at the end of 5, 10, and 15 days. There were 5 replicates of each pair of cultures at each date.

The organisms used included the following: O. graminis (strain S_1); a highly pathogenic strain of Helminthosporium sativum and one of a Fusarium species both

isolated from the basal parts of wheat plants; a yeast; two bacteria - 012 and 015 - from wheat stubble; a bacterium - 05 - known to stimulate the growth of O. graminis in a biotin free medium; a bacterium - ORS - capable of increasing the sporulation of a fungus isolated from a lesion on an orange fruit; and a Trichoderma species from the soil.

After inoculation with O. graminis all plates were incubated in the light of the laboratory for 3 weeks. They were then placed in the greenhouse in the diffuse light of a humidity chamber held at 15° - 20°C.

Observations on growth interaction were made at the end of 3 weeks and on perithecial formation after 6 weeks. The results are presented in Table VI and are illustrated in Figures 5 - 9.

The method of describing the growth interaction is the same as that used by Broadfoot (7). Any two colonies are considered as antagonistic or compatible. These two divisions are subdivided in the following manner:

I. Antagonistic

A. A distinct neutral zone between O. graminis and the introduced organism.

B. A distinct line of demarcation between O. graminis and the introduced organism.

II. Compatible

A. *O. graminis* grows over the introduced organism.

B. Introduced organism grows over *O. graminis*.

The table and illustrations show that the *Fusarium* sp., the yeast, and the bacterium ORS were compatible with strain 8 at the three dates of testing. *H. sativum* and strain S_1 were antagonistic and *Trichoderma* completely inhibitive at all three dates.

There was a great difference in the growth reaction between plantings made at different times. This is well illustrated by bacterium OS which overgrew *O. graminis* at 0 days but caused inhibition when planted later. Bacterium 015 overgrew *O. graminis* at 0 days but was inhibited if planted after that.

In spite of the great variation of growth interaction of the various microorganisms at the different dates there were no perithecia produced by *O. graminis*. This provides further support for the suggestion of Davies et al (15) that ability to stimulate sporulation of *O. graminis* appears to be a property possessed by relatively few individual microorganisms. These differences emphasize the importance of age of culture in relation to the production of stimulatory and antibiotic substances, and the resulting

interaction. These results agree in general with those of Slagg and Fellows (78) and Broadfoot (7) who concluded that the interaction of O. graminis and another microorganism under one set of conditions is no criterion of what it may be under other conditions.

Possibly in plate studies similar to those reported here, more organisms could be found which would induce O. graminis to sporulate if the time of the addition of the inoculum of the introduced organism or other conditions were varied.

2. Flask Studies

Red Bobs wheat seedlings were infected according to the flask method previously described using as aseptic methods as possible. Five days after seeding, the same microorganisms used in the previous plate studies were added singly to the flasks by placing a bit of agar culture at the base of each seedling. In one treatment a bit of natural unsterilized soil was introduced. There were 12 replications of each culture treatment and 20 of O. graminis alone. Some flasks were discarded because contamination inhibited infection completely. Eight weeks after seeding, the plants were examined for perithecia. At the same time a bit of soil from each flask was streaked

TABLE VI

THE EFFECT OF TIME OF INOCULATION OF VARIOUS CULTURES ON THEIR INTERACTION WITH *OPHIOBOLUS GRAMINIS* SACC. ON POTATO DEXTROSE AGAR, AND THE EFFECT OF THESE CULTURES ON THE SPORULATION OF *O. GRAMINIS* IN PURE CULTURE AND ON WHEAT GROWING IN FLASKS

	Interaction on agar plates				Perithecial production in flasks						
	No. days plates inoculated after *Ophiobolus*										
Added Cultures	0	5	10	No. perithecia	No. flasks	No. flasks with perithecia	% flasks with perithecia	No. flasks contaminated	No. contaminated, with perithecia	No. not contaminated, with perithecia	Mean pathogenicity rating
Check-strain-8* (Fig. 5)	11-A	11-A	11-A	0	19	8	42	4	0	8	4.0
O. graminis-S_1 (Fig. 5)	1-B	1-A	1-A	0	11	5	45.5	4	3	2	4.6
H. sativum-6 (Fig. 6)	1-A	1-B	1-A	0	9	4	44.5	3	1	3	4.1
Fusarium sp.-6 (Fig. 6)	11-B	11-A	11-B	0	11	1	9	8	1	0	4.0
Yeast (Fig. 7)	11-B	11-A	11-A	0	9	3	33.5	5	2	1	4.3
Bacterium-012 (Fig. 7)	11-A	1-A	1-A	0	9	7	77.5	5	2	5	4.1
Bacterium-0S (Fig. 8)	11-B	1-A	11-A	0	10	3	30	0	0	3	4.3
Bacterium-015 (Fig. 8)	11-B	1-A	1-B	0	9	2	22	2	0	2	4.1
Bacterium-0RS (Fig. 9)	11-A	11-A	11-A	0	10	4	40	5	2	2	4.0
Trichoderma sp. (Fig. 9)	--	z	z	0	10	0	0	0	0	0	0
Contaminated with natural soil	--	--	--	--	11	1	9	9	--	--	3.1

Pathogenicity rating -

0 = non pathogenic
5 = plants killed
1-4 = intermediate stages

*Checks were inoculated with *O. graminis*-8

z = *Ophiobolus* inhibited completely

Figure 5

Association effects of different colonies of Ophiobolus graminis - effect of time of planting of introduced organism on the growth reaction of strain 8 on potato dextrose agar. Plantings left to right at 0, 5, and 10 days.

Upper row - O. graminis - 8 vs. O. graminis - 8. Note compatibility of colonies.

Lower row - O. graminis - 8 (on left) vs. O. graminis S_1 (on right). Note the slight antagonism between colonies and the rough type of growth exhibited by strain S_1.

Figure 6

Association effects of other organisms on Ophiobolus graminis - effect of time of planting of introduced organism on the growth reaction of Ophiobolus graminis - 8 on potato dextrose agar. Plantings left to right at 0, 5, and 10 days.

Upper row - O. graminis - 8 (on right) vs. Helminthosporium sativum - 6 (on left).

Lower row - O. graminis - 8 vs. Fusarium sp. - 6.

Figure 7

Association effects of other organisms on Ophiobolus graminis - effect of time of planting of introduced organism on the growth reaction of Ophiobolus graminis - 8 on potato dextrose agar. Plantings left to right at 0, 5, and 10 days.

Upper row - O. graminis - 8 (on right) vs. yeast (on left).

Lower row - O. graminis-8 (on right) vs. bacterium - 012 (on left). Note compatibility at 0 days, antagonism at 5 and 10.

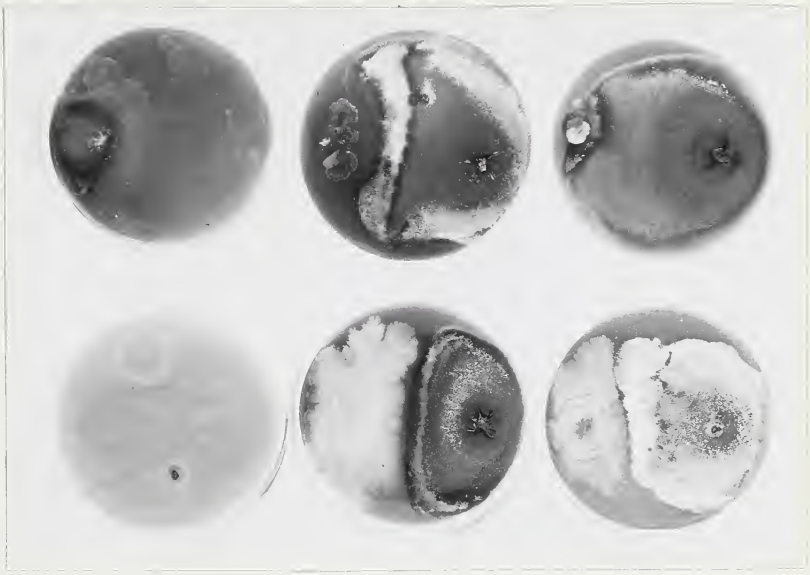

Figure 8

Association effects of other organisms on Ophiobolus graminis - effect of time of planting of introduced organism on the growth reaction of Ophiobolus graminis - 8 on potato dextrose agar. Plantings left to right at 0, 5, and 10 days.

Upper row - O. graminis - 8 vs. bacterium - OS.

Lower row - O. graminis - 8 vs. bacterium - O15.

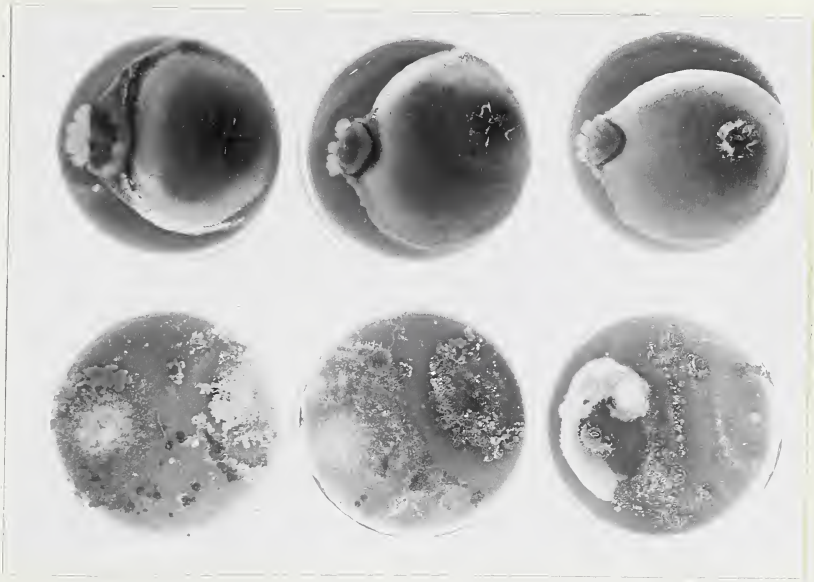

Figure 9

Association effects of other organisms on Ophiobolus graminis - effect of time of planting of introduced organism on the growth reaction of Ophiobolus graminis - 8 on potato dextrose agar.

Upper row - O. graminis - 8 vs. bacterium - ORS. Plantings of bacterium, left to right, at 0, 5, and 10 days.

Lower row - O. graminis - 8 vs. Trichoderma sp. Plantings of Trichoderma, left to right, at 5, 10, and 15 days. Note that O. graminis is overgrown at first two dates of planting.

on an agar slant to determine the sterility of the contents. Usually the introduced organism was reisolated. A summary of the observations made is presented in Table VI.

Of the 9 organisms tested only the bacterium 012 appeared to stimulate greater perithecial production by *O. graminis*. The Fusarium sp. seemed to have an inhibitory effect. The contents of unsterile soil apparently reduced the pathogenicity of *O. graminis* and perithecial production. *Trichoderma* overgrew the flasks and inhibited *O. graminis* completely. Seven of the remaining 8 organisms had little effect on the pathogenicity of *O. graminis*-8, but another strain of *O. graminis* (S_1) seemed to stimulate it.

The results of this experiment do not agree with the suggestion of Davies et al (15) that unsterile conditions tend to favor sporulation. There were fewer perithecia in flasks contaminated either purposely or accidently with a composite microflora than in sterile checks. The importance of individual organisms is emphasized since one bacterium, 012, seemed to increase perithecial formation. This result is comparable to that of Davies (14) who found a bacterium capable of inducing sporulation in pure culture. Davies' bacterium and 012 could not be compared as the former was not available at the time these studies were conducted.

Experiment 3

Possibly the microflora associated with the wheat plant in the field may induce sporulation. Furthermore, perithecia are often found late in the season on infected dead wheat stubble and it is possible that some nutrient or the microflora present at that time may have a particular stimulatory effect.

In order to investigate these possibilities, wheat stubble was gathered from a field in the fall and planted in association with diseased wheat seedlings in small cardboard boxes in the greenhouse. The diseased seedlings were harvested from artificially inoculated soil and washed free of soil. Some were then wrapped around the unwashed stubble before planting in the boxes containing sterile soil, while others were planted alone. The boxes were placed in a humidity chamber in the greenhouse. The soil was kept saturated with water. All plants died in about 3 weeks time. At the end of 8 weeks, the plants were harvested and examined for fruiting bodies.

Perithecia were not observed on any of the plants. Abundant sporulation of Wojnowicia graminis occurred on the wheat stubble.

Evidently under the conditions of this experiment, the wheat stubble or its microflora could supply no stimulatory substance in satisfactory quantities to induce O. graminis to sporulate.

Experiment 4

A further attempt was made to determine the effect of the natural microflora of the soil on sporulation. Twenty-five wheat seeds were planted in pots containing sterile soil artificially inoculated with strain 8. When good infection was apparent, one set of 8 pots was recontaminated by leaching the soil twice with a suspension of natural soil in water. Another set was leached with sterile water.

About 8 weeks after seeding the plants were harvested and examined. At the same time the relative abundance of the microflora of the sterile and recontaminated pots was determined by the dilution plate method.

No perithecia were observed on any of the plants. Approximately the same number of colonies of bacteria and fungi were isolated from the soil in each set of pots but there was a much greater variety of organisms, from the recontaminated soil than from the 'sterile' soil.

This experiment suggests that the mere provision of unsterile conditions in the soil does not necessarily supply the mechanism for perithecial production of *O. graminis*.

Throughout this experiment the relative humidity was not held at a high level and this might explain the lack of sporulation. On the other hand the soil was kept well watered at all times and the plants never dried out.

Discussion

The results of the experiment reported here suggest that unsterile conditions are not necessary for sporulation of *O. graminis* on the host. Perithecia were formed under sterile or relatively sterile conditions. Recontamination of sterile soil or the association of diseased seedlings with unsteriled wheat stubble had no apparent stimulatory effect on fruiting. However, a few individual organisms may be stimulatory - a bacterium was found which seemed to increase fruiting on the host.

Davies (14) found a bacterium which induced perithecial production in pure culture. Possibly in nature perithecial development depends (other conditions being favorable) on the presence of certain individual organisms in sufficient quantities in association with diseased plants.

Any factor such as soil type, pH, and moisture content, which would influence the relative number of these organisms present in the soil or the production of their stimulatory substances would also likely influence perithecial production by O. graminis. This possibility might explain why perithecia are not always found in nature on plants infected with the Take-all fungus. This also might account for the discrepancy of observations made by Davies et al (15) and those made in these studies on the effect of unsterile conditions in the soil on sporulation.

THE EFFECT OF NUTRITION ON SPORULATION

Introduction

The nutritional conditions necessary for reproduction of fungi are not well known. However, the growth requirements are better understood and have been discussed recently in a review by Wolf and Wolf (87). Some of the elements often required by fungi for growth and incorporated into media as inorganic salts include sodium, potassium, phosphorus, magnesium, sulfur, iron, copper, zinc, and boron. The nitrogen requirements may often be satisfied by the inorganic form; at times the organic form is necessary. Usually carbon is supplied as a hexose sugar or as a disaccharide. With some fungi other substances may be required before growth occurs. These may include the vitamins or other growth factors.

Most fungi including O. graminis must produce vegetative growth or mycelia before reproductive bodies may arise. Thus, possibly, any nutritional factor affecting growth will also affect reproduction. However, this

direct association has seldom been proved and there is evidence to show that this supposition does not necessarily apply (3).

The following account includes a fairly extensive review of the literature dealing with the effect of nutrition on the sporulation of the fungi as a whole. This is followed by a description of several studies made at this laboratory on the relationship between nutrition and the development of O. graminis. Although experiments were designed primarily to induce sporulation of the organism, if possible, observations were made on the effect of various nutritional factors on growth and development of perithecia-like black bodies.

Literature Review

Inorganic nutrients

Available information on the effect of the individual elements on sporulation is extremely limited. Asthana and Hawker (2) reported that phosphorous is essential for good growth and perithecial production of

Melanospora destruens. Gilbert and Hickey (26) promoted sporulation of Penicillium notatum in submerged culture by adding iron in small quantities to the solution. Recently, Shu and Johnson (77) have shown that high concentrations of Zn , NH_4NO_3, and KH_2PO_4 retarded spore formation of Aspergillus niger, but increasing the concentration of Mn favored sporulation.

Nitrogen Source

According to Coons (12), widely different classes of compounds may serve as the nitrogen source for pycnidium formation by Plenodomus fuscomaculans.

Leaver and his associates (50) after studying the nutritional requirements of Piricularia oryzae concluded that a large number of diverse nitrogenous compounds are effective in supporting conidial formation but that the amino-acids are required for full activity. This amino-acid requirement is apparently satisfied by glycine alone.

Of several nitrogen sources tested, Westergaard and Mitchell (83) found KNO_3 most satisfactory for the sporulation of Neurospora.

Leonian and Lilly (52) reported that aspartic acid is a satisfactory nutrient for zygospore formation in Phycomyces blakesleeanus. Glycine, arginine, a-alinine, and asparagine by themselves are poor sources of nitrogen but under the influence of succinic acid they induce a large number of zygospores. Ammonium nitrate, however, while greatly stimulating growth completely suppressed spore formation under certain conditions even when all necessary nutrients were present. Leonian (51) found that certain nitrates also reduced pycnidial formation and prevented perithecial production of Valsa leucostoma.

Carbon Source

Hawker and co-workers have presented a series of papers on the sporulation of certain Ascomycetes in relation to their carbon source. Seven of these fungi were essentially similar in their reaction to glucose and fructose. Growth and fruiting increased with an increase in hexose sugars up to a concentration, varying with species, above which fruiting fell off while mycelial growth continued to increase. The response to more complex carbohydrates showed more variation and was of three types: one similar to that

for hexose sugars; a second characterized by a starvation type of growth at a low concentration and a slight increase in both growth and fruiting at higher concentrations; and a third or intermediate type in which growth and fruiting were poor at low concentrations but increased with an increase in carbohydrates until both were good. The type of response of three fungi was correlated with the rate at which sugar was inverted and with the amount of invertase produced (35).

In a later paper (33) it was established that certain di- and polysaccharides are better sources of carbon for fruiting of Melanospora destruens than the hexose sugars. While Miss Hawker considered that this was partly due to a rate of hydrolysis giving favorable concentration of hexoses over a relatively long period, she also presented evidence to show that the readiness with which certain phosphoric esters - e.g. glucose - 1 - phosphate are formed is significant. This worker also suggested that the amount of available energy may be a controlling factor.

Recently, Miss Hawker (34) has proved that phosphoric esters are beneficial to the sporulation of Melanospora. Perithecial production in a synthetic medium was stimulated by the addition of various hexose phosphates.

The stimulatory effect of glucose - 1 - phosphate, of a mixture of monophosphates, and of fructose - 1: 6 - diphosphate were approximately the same.

Westergaard and Mitchell (83) found that glucose or carbohydrates containing glucose are the most effective of the carbohydrates tested for inducing sporulation of Neurospora. High concentrations of sugar were superior to low concentrations.

Natural Products, Growth Factors, and Vitamins

Many fungi require media containing some material of natural or organic origin for growth and reproduction. Sometimes these natural products may be replaced by traces of vitamins or other growth factors.

Robbins and Kavanagh (69) have reviewed the effect of vitamins and growth factors on the development of the filamentous fungi, including sporulation. The phenomena of sexuality of the fungi in relation to these same substances has been discussed by Schopfer (75).

Snyder and Hansen (80) have described the superior value of pea straw agar sterilized by means of fumigation, over heat sterilized media, for the sporulation of Alternaria solani and a Gloeosporium sp.

Leaf juice expressed from Plantanus racemosa leaves and sterilized by filtration through Chamberlain filters was used successfully by Smith and Smith (79) for producing sporulation of four leaf-spotting fungi attacking this plant.

Mrak et al (58) used ground, unpeeled carrots, beets, cucumbers, and potatoes incorporated into a stock medium to induce fruiting of yeasts and other fungi.

Leonian (51) found that malt extract was necessary for reproduction of Valsa leucostoma. According to Shu and Johnson (77) this substance also accelerates spore formation by Aspergillus niger. Deschiens (18) was able to obtain a luxuriant yield of spores by growing certain Hyphomycetes on media containing oat chaff as well as malt extract.

Corticium rolfsii fruited abundantly on onion-proteose peptone and potato dextrose agar when Milthorpe (57) tested these media but the former was the better of the two. Venkatakrishnaiya (82) also succeeded in getting this organism to fruit using an onion asparagine agar.

Benham (6) reported recently that 49 of the 50 strains of Trichophyton rubrum tested, produced typical microconidia on blood agar base, whereas they were formed

by only two of the strains when grown on Sabouraud's honey or dextrose agar. This author suspected that tryptose was the ingredient responsible for spore formation.

Working with Neurospora, Butler et al (8) concluded that biotin is essential for the growth but that other factors are necessary for free production of ascospores. However, Westergaard and Mitchell (83) reported recently that many strains of this organism fruit in a synthetic medium containing biotin. But certain mutants failed to produce perithecia unless certain ingredients such as yeast extract, malt syrup, or acid-hydrolyzed casein had been added to the medium.

Products of natural origin necessary for sporulation may often be replaced by minute traces of one or more of the known vitamins. Asthana and Hawker (2) noted that malt extract was necessary for the fruiting of Melanospora destruens. Miss Hawker (31) later demonstrated that this organism and a number of other unrelated fungi sporulated readily in a medium containing a crude extract of lentils. Later it was shown that biotin was necessary for good growth and thiamin for the production of perithecia by some of these fungi including Melanospora.

Nickerson and Thimann (59) discovered that the conjugation-promoting principle for certain Zygosaccharomyces species was produced by Aspergillus niger and that this principle could be replaced by a mixture of riboflavin and glutamic acid.

Hazen (36) observed that yeast extract is essential for growth and macroconidial formation of Microsporum audouini but that thiamin and pyridoxine could not substitute for this substance.

Miller (56) reported that Venturia inaequalis and Sclerotinia fructicola gave highest yields of conidia on 10 percent malt agar. The best medium for fruiting of Phytophthora infestans was lima-bean agar. The addition of Brewer's yeast, yeast extract, riboflavin, thiamin chloride, and corn steep liquor in varying amounts to potato dextrose agar did not increase sporangial yield by the latter organism.

That biotin is essential for the sporulation of Sordaria fimicola has been shown by Barnett and Lilly (3). These same workers reported that Ceratostomella fimbriata requires thiamin for perithecial formation but possesses no deficiency for biotin, pyridoxine or inositol (4). They also found that

Chaetomium convolutum requires exogenous thiamin and biotin (54). Robbins and Ma (70) found that a Ceratostomella sp. requires thiamin. Recently, Leaver et al (50) have discovered that biotin and thiamin are required for growth and sporulation of Piricularia oryzae.

Agar may contain substances which induce sporulation of certain fungi. Robbins (67) demonstrated that agar contains a growth factor necessary for zygote formation by Phycomyces blakesleeanus. This substance was not replaceable by several of the known vitamins but was found to consist of two factors called Z_1 and Z_2. Factor Z_1 has been identified as hypoxanthine. The same activity is exhibited by guanine. Factor Z_2 remains unidentified (75).

Agar is also known to contain thiamin and biotin. The subject is reviewed by Day (17). She found that different kinds of agar varied in their thiamin content. Difco granulated agar contained about 0.1 μg.* of thiamin per gram and crude shredded agar about 0.5 μg. per gram. Robbins (68) reported that the biotin content of agar also varies with the sample amounting to approximately 0.1 μg. per gram in some cases.

*μg. = microgram = 0.001 mgm.

Nutrient Ratios

The ratio of the concentration of one nutrient to another in the medium seems to influence the sporulation of some fungi. According to Schopfer (75) definite quantities of carbon (maltose) and of nitrogen (asparagine) are necessary for the production of zygotes by Mucor hiemalis. A definite ratio between these two constituents is also required. Above or below a certain ratio, zygotes do not develop.

Recently Barnett and Lilly (3) have emphasized the importance of the vitamin nutrient ratios. They suggest that a proper balance between the amount of biotin, the supply of nutrients, pH, and other factors are prerequisites for normal sexual reproduction in Sordaria fimicola (3). These workers also found that sexual reproduction by Ceratostomella fimbriata occurs only when the ratio of thiamin to the amount of nutrients in the medium is relatively high (4). Similarly for Chaetomium convolutum, the higher the concentration of food in the medium, the greater the amount of biotin and thiamin required for the formation of perithecia (54).

Leonian and Lilly (52) concluded that high or low dextrose-aspartic acid ratios tended to reduce zygote production by Phycomyces blakesleeanus.

On the other hand, Asthana and Hawker (2) demonstrated that sporulation of Melanospora depended on the actual amount of sugar and nitrogen present rather than on the ratio of these two.

Camp (9) observed that fruiting in the slime mold, Physarum polycephalum may be induced by exhaustion of nutrients.

Sporulation of Pyronema confluens begins in fluid cultures according to Robinson (71) only when the available nitrogen is becoming exhausted. Similarly no general development of reproductive structures occurs if the initial concentration of maltose is fairly high. Vegetative growth continues until the concentration of nitrogen and carbon is reduced to a level at which perithecia can be formed.

pH

Hydrogen-ion concentration of the medium may influence the sporulation of fungi. Leach (49) correlated abundant perithecial production by Mycophaerella musicola, the cause of banana leaf spot, with the growth of the host in highly acid soil of pH 4.0 to 4.75. Lilly and Barnett (53) reported that Sordaria fimicola would produce perithecia

in the presence of adequate growth factors only after the pH of the substrate had risen above 6. Similarly, Gardner (22) associated sporulation and elongation of conidiophores of Aspergillus gigantens with pH changes from 4.8 to basic. Anderson (1) found that the sporulation of Gibberella zeae was directly influenced by the initial pH which in time was dependent on the nitrogen source. On the other hand, Asthana and Hawker (2) observed that pH had no effect on sporulation by Melanospora within wide limits.

The Nutrition of O. graminis

Very few studies have been made of the nutrition of O. graminis in relation to growth and fruiting. Fellows (21) concluded that this organism is unable to utilize inorganic forms of nitrogen. Padwick (61) found that a nitrogen-free carrot extract contained a substance capable of stimulating growth in a synthetic nutrient solution. Garrett (24) later observed that O. graminis grew well if a wheat straw extract was provided in place of the carrot extract.

White (85) demonstrated that O. graminis requires biotin and thiamin for mycelial development. Growth occurs only when biotin is present; but more than double the amount of growth results when thiamin is also added. He reported

that about 1000 µg. each of biotin and thiamin are required for optimal growth conditions. Growth factors are present in optimal amounts in extracts of wheat roots, wheat straw, and peptone. The necessity of biotin and thiamin for mycelial development has been supported in a recent paper by Davies et al (15).

The work of Padwick (61), Garrett (24), and White (85), revealed that O. graminis may utilize various inorganic forms of nitrogen in the presence of growth factors and inorganic nutrients and disproved Fellow's earlier work which was conducted in the absence of growth factors. Later White (86) demonstrated that a wide range of compounds are utilized by this organism as a source of carbon and nitrogen for respiration and assimilation. He reported that the optimal concentration for the nitrogen source is equivalent to 200 mgs. nitrogen per litre and the carbon source is equivalent to 2.0 percent glucose when nitrogen is supplied as KNO_3, NH_4NO_3, glycine, or asparagine; but the concentrations are 200 mgs. of nitrogen per litre and 1.0 percent glucose when nitrogen is supplied as a mixture of amino acids or peptone.

White (86) also observed that there are differences in assimilability of compounds by O. graminis. When carbon and nitrogen are present in optimal amounts these differences are due to anabolite efficiency values of the compounds

which condition the growth rate rather than the maximal amount of growth of the fungus and this (in addition to the biotin and thiamin requirement) is a third factor limiting the growth of O. graminis in synthetic solutions. The efficiency values are optimal for growth when nitrogen is supplied as a mixture of amino acids or peptone, and carbon is supplied as glucose in optimal amounts. White (86) conducted his experiments in liquid media and made no reports on the effect of various nutrients on sporulation.

Various natural media have been used successfully for inducing sporulation of this organism. Kirby (47) obtained perithecia on crushed wheat agar and sterilized sweet clover and wheat stems. Davis (16) found that string bean agar was a suitable medium. Of several media tested by Russell (72), ground oat hulls was the only satisfactory substrate for perithecial formation.

The Effect of Various Nutritional Factors on the Development of *O. graminis*

Introduction

The preceeding literature review reveals the importance of nutrition in the sporulation of fungi. As mentioned previously, Davies (14) had found a bacterium which would induce sporulation of *O. graminis* in pure culture. Since certain microorganisms are known to stimulate sporulation of other fungi by supplying them with required nutrients, it is possible that Davies' bacterium was able to supply *O. graminis* with some specific nutrient for sporulation. Thus the following studies were conducted in an attempt to determine the nutritional conditions necessary for the sporulation of *O. graminis* in artificial culture.

Methods

Strain 8 of *O. graminis* was used throughout these experiments. At times S_1 served as a check.

Unless otherwise stated, the basic nutrient solution had the following composition:

Dextrose	20gm.
KH_2PO_4	1gm.
Na_2HPO_4	3gm.
$MgSO_4$	2gm.
$NaNO_3$	2gm.
$FeCl_3$	trace (0.001 gm)
$NaBO_2$	trace
$CuSO_4$	trace
$ZnSO_4$	trace
$(NH_4)_2MoO_4$	trace
Agar	15gm.
Distilled water to make	1000 ml.

Other substances were added according to the treatment. The sources of the chemicals used in these studies were as follows:
sugars - Difco, Eastman Kodak, and Baker's; amino acids - Smaco and Eastman Kodak; thiamin hydrochloride and nicotinic acid - Merck; biotin, p-amino benzoic acid, and folic acid - General Biochemicals; various extracts and peptone - Difco.

All glassware was washed thoroughly with trisodium phosphate or dicromate-sulfuric acid cleaning solutions. Experiments were conducted in Petri plates, test-tubes or

Erlenmeyer flasks. Each plate or flask contained 25 ccs. of medium while agar slants had 10 ccs. All media were sterilized at 120°C for 20 minutes. The pH was adjusted to 6.7 before autoclaving. The inoculum consisted of a bit of mycelium from a fresh culture growing on potato dextrose, basic nutrient, or water agars.

There were 5 to 10 replicates for each treatment. Some experiments were repeated 2 or 3 times. Cultures were incubated in a humidity chamber at 15° - 20°C. in diffuse light in the greenhouse. One experiment on growth alone was conducted in a 20°C constant-temperature chamber in the dark.

Ratings of growth were made at the end of 14 days. On agar these were based on the diameter of the colony and the density of growth. Development in liquid media was measured by the dry weight of mycelium. Examination for the presence of perithecia was made at the end of 8 weeks.

The Effect of Vitamins and Growth Factors

In this experiment, thiamin was added at the rate of 10,000 µg. per litre of solution and other vitamins at

the rate of 100 µg. The extracts and peptone were supplied at a concentration of 20 gm. per litre.

The experiment was conducted in triplicate; with strains 8 and S_1 in Petri plates and with strain 8 in test tubes. Folic acid and p-amino benzoic acid were included only in the Petri plate study with strain 8. The inoculum was taken from cultures growing on basic nutrient agar.

The results of the studies with strain 8 are presented in Table VII and Figures 10 and 10a.

The necessity of biotin for growth and of thiamin for further vegetative development of O. graminis was confirmed. The further addition of niacin, riboflavin, calcium pantothenate, pyridoxine, folic acid, p-amino benzoic acid inositol, and vitamin C singly and in combination had no obvious effect on growth. Sufficient growth substances are evidently supplied in malt, yeast, and string bean extracts and in peptone. Growth is slightly better in the presence of the extracts than with peptone. Mycelium in the presence of inositol was dark in color as contrasted to the white fluffy growth on the other media.

Some growth occurred on the basic medium and on water agar. Evidently O. graminis is able to derive enough

nutrients and growth factors from agar to produce some growth but not enough to cause discrepancy in the present studies.

Mature perithecia were not observed on any of the media. Perithecia-like black bodies were formed only when biotin and thiamin were both present. The further addition of pyridoxine, folic acid, and p-amino benzoic acid seemed to increase slightly the production of these bodies when added singly but not when added in combination with the other vitamins. Black bodies failed to form in the presence of malt and yeast extracts and peptone. Of all the substances tested, string bean extract seemed to be the most favorable for their production.

TABLE VII

THE EFFECT OF VARIOUS VITAMINS AND GROWTH FACTORS ON THE DEVELOPMENT OF *O. GRAMINIS* (STRAIN 8)

Medium		Growth (14 days)		Relative number	
No.	Description	Diameter (cms.)	Density	black bodies	Perithecia
1	1.5% water agar	?	0.5	0	0
2	Basic	?	1	0	0
3	Basic + biotin	30.0	2.5	0	0
4	Basic + thiamin	?	1.5	0	0
5	Basic + biotin + thiamin	35.7	5	++	0
6	Medium 5 + niacin	38.4	5	++	0
7	5 + riboflavin	37.1	5	++	0
8	5 + calcium pantothenate	36.5	5	++	0
9	5 + pyridoxine	37.3	5	+++	0
10	5 + folic acid	38.0	5	+++	0
11	5 + p-amino benzoic acid	38.1	5	+++	0
12	5 + vitamin C	36.6	5	+++	0
13	5 + inositol	34.9	5	++	0
14	Basic + all vitamins above	36.9	5	++	0
15	Basic + 2% malt extract	34.4	9	0	0
16	Basic + 2% yeast extract	32.1	10	0	0
17	Basic + 2% string bean extract	33.2	10	++++	0
18	Basic + 2% peptone	36.3	8	0	0

? = Growth was so sparse that colony diameter could not be measured.

Figure 10

Effect of vitamins on the development of Ophiobolus graminis.

Upper row, left to right - water agar, basic nutrient medium, basic + biotin, basic + biotin + thiamin (medium 5), medium 5 + niacin, 5 + riboflavin.

Lower row, left to right - 5 + Ca. pantothenate, 5 + pyridoxine, 5+ folic acid, 5 + p-amino benzoic acid, 5 + vitamin C, 5 + mixture of vitamins, 5 + inositol.

Note the black bodies in several cultures and the dark colored mycelium in the presence of inositol.

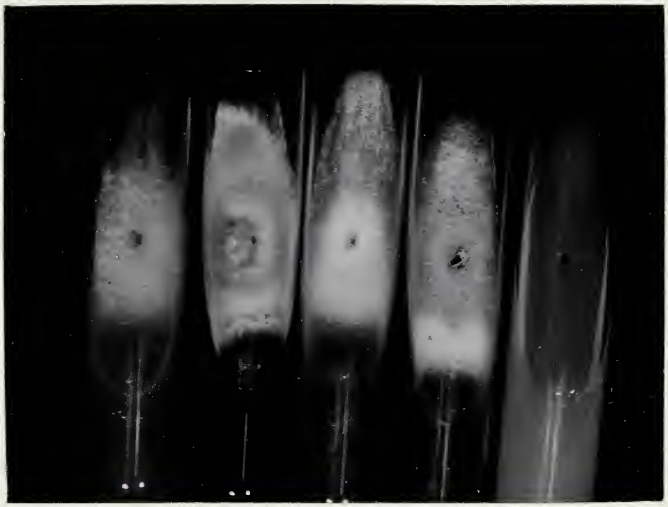

Figure 10a

Effect of growth factors on the growth of Ophiobolus graminis.

Left to right - Basic nutrient medium + peptone, basic + string bean extract, basic + yeast extract, basic + malt extract, and basic alone.

The Effect of Concentration of Biotin and Thiamin

Inasmuch as only one concentration of biotin and thiamin was used in the previous experiment, it was decided to test the action of other concentrations. The basic medium was supplemented with various concentrations of these two vitamins and their effect on the development of *O. graminis* in Petri plates was noted and recorded in Table VIII. The effect of concentration of biotin and thiamin on growth is shown in Figures 11 and 12.

The results further illustrate the necessity of biotin for the growth of *O. graminis*. Growth was very poor on basic agar medium but there was fair development when biotin was present at the rate of 0.1 μg. per litre. Although there was good growth when biotin was supplied in concentrations of 1.0 to 100 μg. per litre, 100 μg. seemed to provide optimum conditions. *O. graminis* was inhibited at a concentration of 1000 μg. per litre.

Growth was much better when thiamin was also added to the medium. A concentration of 10,000 μg. per litre seemed to induce maximum development. Concentrations greater than this had no further stimulatory effect.

TABLE VIII

THE EFFECT OF CONCENTRATION OF BIOTIN AND THIAMIN ON THE DEVELOPMENT OF *O. GRAMINIS*

Concentration (µg/l.)		Growth		Relative No. black bodies	Peri-thecia
Biotin	Thiamin	Diameter (cms.)	Density		
0	0	?	1	0	0
0	1000	18	3	0	0
0.1	1000	65	3.5	0	0
1.0	1000	77	7	+	0
10	1000	86	7	++	0
100	1000	87	8	+++	0
1000	1000	42	9	+++	0
100	0	68	7	0	0
100	100	75	8	+++	0
100	1000	87	8	++++	0
100	10,000	90	10	+++	0
100	100,000	88	10	+++	0
100	1,000,000	91	10	+++	0

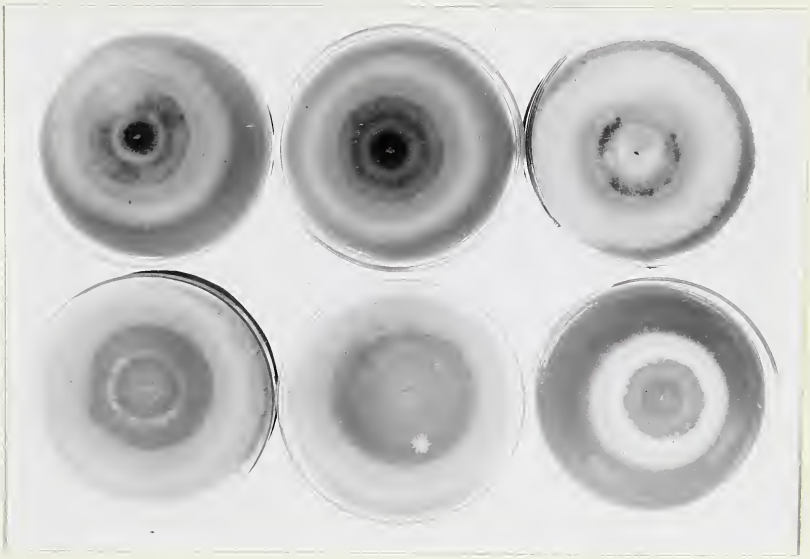

Figure 11

Effect of concentration of biotin on growth of Ophiobolus graminis.

Concentration of biotin in μg. per litre, left to right -

Upper row - 0, 0.1, and 1.0.

Lower row - 10, 100, 1000, (note inhibition).

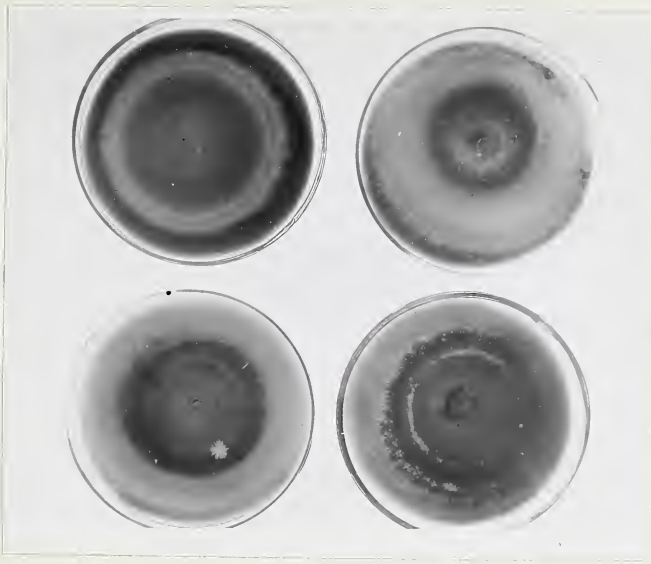

Figure 12

Effect of concentration of thiamin on the growth of *Ophiobolus graminis*.

Concentration of thiamin in µg.per litre, left to right -

Upper row - 0 and 100; lower row - 1000 and 10,000.

No perithecia were observed at any concentration of biotin and thiamin. No black bodies were formed if thiamin was absent from the medium but increasing the concentration of biotin with thiamin present increased the relative number of these bodies produced. None were formed below a concentration of 1.0 µg. of biotin per litre. Concentrations of biotin and thiamin above 100 µg. per litre did not stimulate their formation further.

In a study of the effect of the interaction of outdoor conditions and concentration of vitamins on the development of *O. graminis* the above experiment was repeated placing the cultures outdoors where they were subjected to diffuse daylight and the alternating temperatures of midsummer. These conditions had no more effect on *O. graminis* than was observed in the previous greenhouse studies.

The Effect of Concentration of Nutrients

An attempt was made to determine the effect on the development of *O. graminis* of reducing the concentration of the nutrients while holding the concentration of the growth factors constant. A series of media were prepared with each of the following dilutions of nutrients: undiluted,

1/2, 1/4, 1/8, 1/16, 1/32, 1/64. Biotin and thiamin were added to each of these dilutions at the rate of 100 and 10,000 µg.per litre respectively. A bit of culture growing on water agar served to start the colonies in these Petri plate studies.

TABLE IX

THE EFFECT OF CONCENTRATION OF NUTRIENTS ON THE DEVELOPMENT OF *O. GRAMINIS*

Dilution of nutrients	Growth		Production of black bodies (8 weeks)	Perithecia
	Diameter (cms.)	Density		
0 (No vitamins)	tr	1	0	0
0 (Vitamins present)	80.5	10	++	0
1/2 "	74.0	6	++	0
1/4 "	67	2.5	0	0
1/8 "	62.5	1.5	0	0
1/16 "	63	very low	0	0
1/32 "	58.5	very low	0	0
1/64 "	51.5	very low	0	0

The results presented in Table IX show that diluting the nutrients greatly reduces the growth of O. graminis. Growth was poor if the concentration of nutrients was reduced to 1/4 of the original. Furthermore, black bodies were not formed below the 1/2 level. No perithecia were observed at any dilution.

The results also show that biotin and thiamin are ineffective as growth factors if adequate nutrients are not supplied. This suggests that the role of these two vitamins added in minute quantities is not in the manner of a nutrient but more in the form of a catalyst or some other similar agent acting on the nutrients in the medium in the production of substances necessary for the growth of the organism.

The Replacement of Biotin by Aspartic Acid

A number of reports have appeared indicating that biotin and aspartic acid are interchangeable as growth factors for certain fungi and bacteria. Beadle and Tatum (5) noted that aspartic acid reduces the biotin requirement of Neurospora and Schopfer (76) found that a similar relationship holds for Phycomyces blakesleeanus. Recently, a report

by Perlman (63) indicates that the addition of this same amino acid to a synthetic medium also markedly reduced the biotin requirement of Memoniella echinata. Furthermore, Koser et al (48) were able to demonstrate that for Torula cremoris, aspartic acid could replace biotin. Conversely, Stokes et al (81) observed that biotin can completely substitute for aspartic acid in the growth of Lactobacillus arabinosus, Streptococcus faecalis and related organisms. They concluded that biotin participates in the synthesis of aspartic acid. Recently, Potter and Elvehjem (64) have also noted that aspartic acid and biotin are almost interchangeable in the nutrition of L. arabinosus.

The ability of aspartic acid to substitute for biotin as a growth factor for O. graminis in the absence of thiamin was investigated in these studies. Aspartic acid at the rate of 1 gm. and biotin at the rate of 100, 500, and 1000 µg. were added singly to a litre of basic nutrient medium. The experiment was conducted in liquid media in flasks. The results presented in Table X indicate that aspartic acid may almost completely substitute for the biotin requirement of this organism. They also support the previous observation that biotin at concentrations over 100 µg. per litre may inhibit the growth of O. graminis.

TABLE X

UTILIZATION OF BIOTIN AT VARIOUS CONCENTRATIONS AND ASPARTIC ACID AS GROWTH SUBSTANCES BY *O. GRAMINIS*

Medium	Dry weight of mycelium in mg. (21 days)
Basic	9.9
Basic + Biotin (100μg/l.)	47.4
Basic + Biotin (500μg/l.)	43.8
Basic + Biotin (1000μg/l.)	41.4
Basic + Aspartic acid (1 g/l.)	36.4

Carbon Source

Since the previous experiments were conducted using only dextrose as the carbon source and since perithecial formation was lacking in these cases, it was thought advisable to test the effect of other carbohydrates on the development of *O. graminis*. In this experiment, growth substances were supplied as wheat-stem extract which was prepared by autoclaving 200 grams of wheat stubble with a litre of water for 20 minutes. The resulting extract was decanted off, filtered, and made up to a litre volume with

water. This extract replaced the water in the basic nutrient medium which was otherwise unchanged except for the carbon source.

TABLE XI

THE EFFECT OF VARIOUS CARBON SOURCES ON THE DEVELOPMENT OF O. GRAMINIS

Carbon source	Growth rating (14 days)	Relative No. black bodies (8 weeks)	Perithecia
dextrose	8	0[1]	0
starch	7	0[1]	0
xylose	7	0[1]	0
inulin	2	0	0
maltose	10	0[1]	0
d-mannose	8	0	0
laevulose	8	0[1]	0
d-galactose	6	0[1]	0
lactose	7	0[1]	0
sucrose	6	0[1]	0
None	1	0	0

[1] = Few poorly developed black bodies.

Figure 13

Effect of carbon source on the growth of Ophiobolus graminis.

Carbon source added to basic nutrient medium with wheat-stem extract as the growth factor source, left to right -

Upper row - check, inulin, sucrose, d-galactose, xylose, starch.

Lower row - check, lactose, dextrose, d-mannose, laevulose, maltose.

Table XI and Figure 13 show that maltose was the best carbon source for the growth of O. graminis at the end of 14 days and inulin was the poorest source. Dextrose, d-mannose and laevulose were intermediate and induced better growth than starch, xylose, d-galactose, lactose and sucrose.

No perithecia or fully-formed black bodies developed on any of the media. However, with several carbon sources, small black specks appeared in the cultures. These may be similar to the perithecia-like black bodies formed in the previous experiments but at a more immature stage of development.

Concentration of Dextrose

In all previous experiments, dextrose and the other carbon sources were supplied at the rate of 2% by weight of the medium. Previous work by White (86) had shown that this was the optimum level for growth of the organism in the presense of inorganic nitrogen. To test the effect of other concentrations of dextrose on the general development of O. graminis this sugar was added in varying quantities to the basic nutrient and wheat-stem

agar medium used in the previous experiment. This test was made using agar slants inoculated with a culture from basic nutrient agar.

TABLE XII

THE EFFECT OF CONCENTRATION OF DEXTROSE ON THE DEVELOPMENT OF *O. GRAMINIS*

% concentration of dextrose	Growth rating (14 days)	Relative no. black bodies (8 weeks)	Perithecia
0	1	0	0
0.5	6	0	0
1.0	7	0	0
1.5	7	0	0
2.0	8	0[1]	0
5.0	7	++	0

[1] = Few poorly developed black bodies.

The results presented in Table XII and illustrated in Figure 14 show that dextrose at the rate of 2% is optimum for mycelial development but that a wide range of concentrations induce satisfactory growth under the conditions of this test.

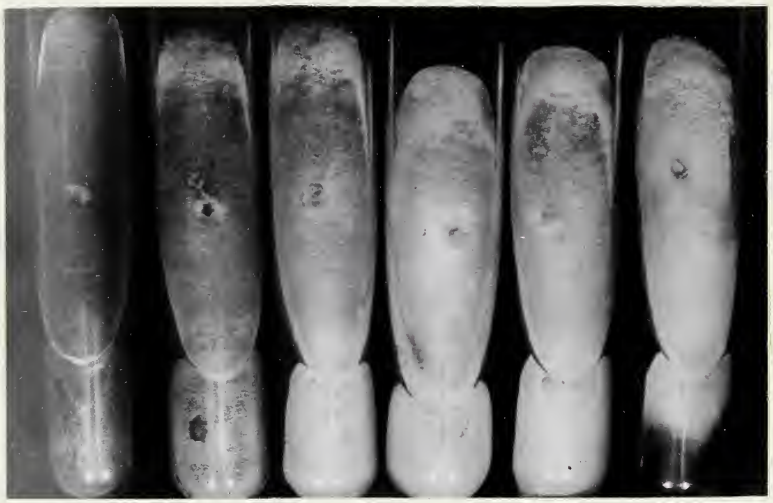

Figure 14

Effect of concentration of carbon source (dextrose) on the growth of Ophiobolus graminis

Concentration of dextrose in percent, left to right - 0, 0.5, 1.0, 2.0, and 5.0.

With a concentration of 5% dextrose, typical perithecia-like bodies similar to those formed in other experiments appeared in great numbers in the cultures by the end of 8 weeks. As in the previous experiment tiny black specks developed at the 2% level of dextrose but none were observed below this concentration. These results show that concentration of carbon source may greatly affect the development of these perithecia-like bodies. These bodies which developed at the 2% level of dextrose on this medium where growth substances were supplied as wheat-stem extract may be similar to those appearing at the 5% level on the same medium and at the 2% level on media containing biotin and thiamin. They are possibly at an earlier stage of development. This possibility was not investigated further.

Nitrogen Source

As previously reported by White (86) the anabolite efficiency values for O. graminis are optimal for growth when nitrogen is supplied as a mixture of amino acids or peptone, and carbon as glucose at optimal concentrations. In the light of the foregoing and inasmuch as $NaNO_3$ was the

nitrogen source in all of the experiments reported thus far it was thought advisable to investigate further the effect of various other nitrogen sources on the development of this fungus.

$NaNO_3$ was replaced by several nitrogen sources at the rate of 0.2% by weight in the basic medium. Biotin and thiamin were added at the rate of 100 and 1,000 µg. per litre respectively. Test-tube slants containing the various media and prepared in the usual manner were inoculated with basic nutrient agar cultures. The results are presented in Table XIII and Figure 15.

Under the conditions of this experiment, peptone and asparagine were the best sources of nitrogen for growth of O. graminis at the end of 14 days. A mixture of 13 amino acids was more effective than any of the individual acids used alone with the exception of d-glutamic acid. The poorest nitrogen source tested was dl-threonine. $NaNO_3$ was intermediate in effect. These results disagree slightly with the findings of White (86) who reported that peptone or an amino acid mixture was superior to asparagine as a nitrogen source.

No perithecia were produced in any of the media. $NaNO_3$ and dl-serine were the only nitrogen sources which induced the production of the perithecia-like black bodies.

TABLE XIII

THE EFFECT OF VARIOUS NITROGEN SOURCES ON THE DEVELOPMENT OF O. GRAMINIS

Nitrogen source	Growth rating (14 days)	Relative no. black bodies (8 weeks)	Perithecia
None (ck.)	1	0	0
$NaNO_3$	5	++	0
asparagine	10	0	0
peptone	10	0	0
aspartic acid	5.0	0	0
l-tryptophane	3.0	0	0
methionine	4.0	0	0
l-histidine	6.0	0	0
phenylalanine	4.5	0	0
dl-isoleucine	3.5	0	0
dl-threonine	2.0	0	0
d-arginine	7.0	0	0
dl-valine	4.5	0	0
l(-)cystine	2.0	0	0
d-glutamic acid	8.0	0	0
l-proline	5.5	0	0
dl-serine	5.5	++	0
Above 13 amino acids in combination	7.5	0	0

Figure 15

Effect of nitrogen source on the growth of Ophiobolus graminis.

Nitrogen source, left to right -

Upper row - check, $NaNO_3$, asparagine, peptone, l-proline, methionine, phenylalanine, dl-isoleucine.

Lower row - l-tryptophane, l-cystine, l-histidine, dl-serine, dl-threonine, d-glutamic acid, dl-valine, d-arginine, mixture of amino acids.

pH

It has been well established that *O. graminis* will grow in synthetic solutions over a wide range of pH. However, since this factor may influence the sporulation of other fungi, it may also play a part in perithecial production by *O. graminis*. This possbility was investigated in the following experiment.

Potato dextrose agar was adjusted with HCl or NaOH to various pH levels. Readings were made with a Beckman glass electrode pH meter before and after sterilization and after the fungus had grown on the media for 6 weeks. These readings are presented in Table XIV.

The pH values of acid media increased as a result of autoclaving while those of basic media were lowered. Hydrogen-ion concentration also tended toward neutrality under the action of the growing organism.

A wide range of initial pH values had no deleterious effect on growth of *O. graminis*. Neither perithecia nor perithecia-like black bodies were observed at any level. These results support the conclusions of other workers that pH has no effect on the development of *O. graminis* over a wide range.

TABLE XIV

THE EFFECT OF GROWTH OF *O. GRAMINIS* AND HEAT STERILIZATION ON THE pH OF POTATO DEXTROSE AGAR

Initial pH	pH after sterilization	pH after 6 weeks growth of Ophiobolus
3.1	4.5	6.0
4.4	5.4	6.0
6.0	6.0	6.1
7.5	7.5	6.8
9.0	8.4	6.8

Discussion

The foregoing studies on the effect of various nutritional factors on the development of *O. graminis* in synthetic culture have provided definite information on the growth requirements of this organism. It was found that biotin is essential for growth and thiamin for further development thus supporting the previous observations of White (85) and Davies et al (15). Several substances of

plant or animal origin may satisfy this requirement. Even agar seems to supply small amounts of these growth factors.

The inhibiting effect of biotin at the concentration of 1000 μg. per litre observed in these studies does not agree with White's observation that this concentration was optimum for growth. Possibly the different physical conditions in the liquid media of White and the solid media of most of the present studies may account for this disagreement. However, one experiment reported here suggests that there is also inhibition at 1000 μg. per litre in liquid media. It is interesting to point out here that Schopfer (75) reported that excessive dosages of thiamin inhibited the growth of certain Rhizopus spp. but that this was dependent on certain physical conditions of the culture and that one nitrogen source prevented or retarded this inhibiting action.

Increasing the concentration of thiamin above a certain level had no further stimulatory effect on growth of O. graminis but caused no inhibition. These contrasting effects of high concentrations of biotin and thiamin coupled with the fact that biotin must be provided before thiamin is effective, suggests that these two substances have different functions in the physiological activity of O. graminis and that the function of the thiamin is dependent on the presence of biotin.

According to Schopfer (75) thiamin is an essential constituent of coenzymes. The loss of the ability to synthesize thiamin thus means that vital enzymatic reactions cannot be carried out unless this vitamin is supplied as an exogenous growth factor. Recently the role of biotin has been linked up with the production of aspartic acid by microorganisms. It is interesting that aspartic acid may partially replace the biotin requirement of O. graminis. Further studies are necessary before the relationship between biotin, thiamin, and aspartic acid in the physiology of this organism, may be understood.

It is unlikely that the ability of aspartic acid to replace the biotin requirement of Ophiobolus is a characteristic of many amino acids. Fellows (21) tested the effect of several amino acids including aspartic acid on the growth of O. graminis in a synthetic medium similar to that used here but lacking biotin. None of the amino acids tested could induce growth.

Several other vitamins are unable to further stimulate growth of O. graminis when biotin and thiamin are present at optimum concentrations. However, yeast, malt, and string-bean extracts and peptone are able to replace these two growth factors and induce more growth than

occurs when the vitamins are present in optimum concentrations. White (85) observed the same phenomenon and later demonstrated (86) that this was the result of the anabolite efficiency values of these compounds which increases the growth rate rather than the maximal amount of growth of the fungus.

Inulin is a poor source of carbon for the growth of O. graminis which agrees with Miss Hawker's observations on the relationship between this substance and a starvation type of growth by Melanospora destruens. Best growth of O. graminis was obtained with maltose but all of the sugars tested induced good growth.

The growth reaction of O. graminis to different concentrations of dextrose observed here is in contrast to the reaction of several Ascomyetes to glucose and fructose as observed by Hawker and Chaudhuri (35). O. graminis grows well over a wide range of concentrations (0.5 - 5.0 percent) whereas growth of their organisms increased with increase in hexose sugars up to a concentration of 10 percent or more.

White (86) pointed out that the growth rate of O. graminis varied according to the complexity of the nitrogen compound (the more complex the compound the greater the rate of growth)

but the maximal growth was approximately the same in all substrates. He noted that 12 to 14 days was needed for maximum development with peptone and asparagine, 17 days with glycine, and 23 days with $NaNO_3$. Furthermore, White suggested that O. graminis probably has an unspecialized nutrition in relation to a supply of nitrogen. Considering the present studies in the light of White's observations one would expect that all of the amino acids tested would induce better growth than $NaNO_3$ at the end of 14 days since they are more complex nitrogen sources. But this is not the case for l-tryptophane, methionine, phenylalanine, dl-threonine, dl-valine, l(-)cystine and dl-serine. This would indicate that for growth O. graminis may utilize certain nitrogen sources much better than others. It would be unwise to draw definite conclusions from the results of the single experiment reported here; yet until further information is provided on this matter the suggestion that O. graminis has an unspecialized nutrition in relation to nitrogen requirements for growth should be considered with caution.

In these studies none of the nutritional conditions provided induced ascospore formation by O. graminis. This would suggest that sexual reproduction of this organism is dependent on some other factor or combination of factors on a highly specific nutritional requirement not provided here.

If the perithecia-like black bodies formed throughout these studies are considered to have any relationship to true perithecia, it is logical to conclude that *O. graminis* has certain specific nutritional requirements for perithecial formation. Production of the black bodies was conditioned by the presence in the culture medium of certain specific growth factors at definite concentrations. Their occurrence was also dependent on the presence of certain specific nitrogen sources and definite concentrations of nutrients.

There is the possibility that some of the nutrients used in the present studies were ineffective in inducing sporulation because they were inactivated by the sterilizing process of heating media for 20 minutes at 120°C. Hawk et al (30) state that thiamin, biotin, nicotinic acid, pyridoxine hydrochloride, folic acid and pantothenic acid are all stable when so treated. Patton and Hill (62) have recently shown that the autoclaving of media containing glucose may inactivate to some extent the B complex vitamins and certain amino acids. The latter include lysine, arginine, and tryptophane. Riesen et al (66) observed that sterilization by autoclaving had varying destructive effects upon the activity of cystine and cysteine,

depending upon the organism. These latter workers report that most amino acids added prior to the autoclaving process may be recovered satisfactorily. It seems unlikely from the foregoing reports that the sterilization process would have any serious inactivating effect on most of the substances used under the conditions of the experiments reported here. Thus inactivation probably does not account for the inability of these substances to induce sporulation of O. graminis in artificial culture in these studies.

GENERAL DISCUSSION

Previous to the initiation of these studies a bacterium had been found in this laboratory which would induce sporulation of *O. graminis* in pure culture (14, 15). Also Garrett (25) had suggested but had supplied no experimental proof that the other microorganisms in the soil might influence sporulation of this organism on the host. Other investigators felt that certain physical factors including light, temperature and moisture might play important roles. Furthermore it had been established that *O. graminis* is homothallic but that there remained the possibility that one strain might supply some stimulus to another strain in pure culture which might induce sporulation.

O. graminis sporulates fairly readily on the host plant if proper physical conditions are supplied. Possibly Davies' bacterium and the host plant are able to furnish *O. graminis* with substances necessary for sporulation. However, in the present studies, none of the vitamins, growth factors, nitrogen and carbon sources added to a basic nutrient medium in various concentrations and combinations was able to replace the effect of the host or Davies' bacterium. Assuming that the black bodies observed in these studies are immature

perithecia it is clear that nutrition plays a significant role in the sporulation of O. graminis.

These and other studies have demonstrated that many individual microorganisms are unable to induce sporulation of O. graminis in artificial culture. Either few individuals have the stimulating ability or pure culture conditions do not provide the proper relationship between the introduced organism and O. graminis for stimulation to occur. The same microorganism may have different effects on the growth of O. graminis at different stages of development. Since a similar relationship may hold sporulation this should receive further study.

Davies (14) found that only the living bacterium was able to induce fruiting of O. graminis in artificial culture. A sterile, staled liquid medium in which the bacterium had grown was ineffective. Possibly the bacterium must be supplied with some stimulation from O. graminis before it may be stimulatory itself. O. graminis might react to any stimulation at only a definite stage of development. More than one substance may be required in proper sequence for perithecial production. In the latter case, it is likely that only a few individual microorganisms would be able to meet such a demand. However, in nature all the conditions

suggested above might be supplied more easily by several microorganisms in association with *O. graminis* and account for the fact that this organism sporulates more readily in nature than in artificial culture. Davies et al (15) concluded that *O. graminis* sporulated more abundantly on wheat plants growing in unsterile soil than in sterile. This conclusion was not borne out by the present studies which suggested the importance of individual organisms. However, it should be appreciated that in such studies it is difficult to duplicate environmental conditions. Since the microflora of the soil varies greatly with the sample it is unlikely that the types and number of microorganisms present in any two experiments would be identical. Furthermore, discrepancies might arise from the biological variation of different individual plants and varieties of the host and of different strains of the fungus. Further studies are required before sufficient information will be available to explain the effect of other organisms on the sporulation of *O. graminis*. These should include the growing of two or more different organisms in association with *O. graminis* at the same time in an attempt to approach the more matural conditions of the soil.

These present studies supplied little information on the role that physical factors play in the sporulation

of O. graminis. Light is evidently important at least to the extent of playing a part in the maturation of perithecia. Definite information on the effect of the physical factors on sporulation will probably not be available until after a technique is devised for inducing sporulation on a medium other than the host plant. By present methods physical conditions acting on the microorganisms also act on the substrate which does not allow the investigator to hold all the conditions uniform while any individual physical factor is under study.

In the light of present knowledge, it seems definite that O. graminis is homotallic and that under the proper conditions single strains may sporulate in the absence of other strains. However, there is often a growth interaction between colonies of different strains in pure culture. Such an interaction might increase perithecial formation once it was initiated. This explanation could possibly account for Kirby's (46) conclusions that + and - strains must be grown together before sporulation of O. graminis will occur.

These studies have shown that independent factors may be important in inducing fruiting. However, none was found which governed the process. Sporulation might depend on a proper balance between several factors. The importance of different physical, nutritional and microbiological conditions in various relationships warrants further study.

SUMMARY

1. The effect of physical, nutritional and biological factors on the fruiting and to some extent on other phases of development are reported in the present study.

2. The association of two colonies of the same or different strains of *O. graminis* either in artificial culture or on the host did not induce perithecial formation.

3. Colonies of different strains were moderately growth-antagonistic, a phenomenon not exhibited by colonies of the same strain.

4. Single strains sporulated on several occasions on wheat seedlings, supporting the view of other workers that *O. graminis* is homothallic.

5. Although highly pathogenic strains tended to be more fertile than less pathogenic strains, one highly pathogenic isolate exhibited only slight sporulating ability.

6. Certain strains were still fertile after growing in pure culture for several years.

7. Two strains while growing on agar produced spermagonia-like bodies which at times contained tiny spore structures

similar to spermatia. Other strains produced black perithecia-like bodies under certain nutritional conditions.

8. A method of inducing the formation of perithecia on the host plant was developed and used repeatedly with success. This consisted of growing wheat seedlings in small flasks containing soil artificially infested with O. graminis. Three methods suggested by other workers were unsuccessful when tested.

9. Mature perithecia developed on the host only under conditions of natural daylight, high humidity and relatively cool temperatures (15°C). These physical conditions were maintained throughout most of the studies reported here.

10. Fruiting did not occur in agar cultures exposed to outdoor environment for several weeks.

11. Light was found to be necessary for the maturation of perithecia. Time of exposure had no effect on the number of fruiting bodies formed or on ascospore length.

12. The organism failed to fruit on artificially-inoculated wheat plants growing outside in soil having a high moisture content.

13. Ninety-eight microorganisms, including 59 bacteria,

24 actinomycetes, and 14 fungi failed to stimulate fruiting of *O. graminis* when associated with the latter in artificial culture on agar.

14. Eight microorganisms added to potato dextrose agar cultures of *O. graminis* did not induce fruiting when planted 0, 5 and 10 days after *O. graminis*. A *Trichoderma* sp. was also ineffective at 5, 10 and 15 days and overgrew *O. graminis* at all three dates. There was a great difference in the growth reactions between plantings made at different dates.

15. Eight of the above organisms did not stimulate fruiting of *O. graminis* when introduced singly into sterile flasks containing diseased wheat seedlings growing under relatively sterile conditions.

16. One bacterium (012) appeared to appreciably increase perithecial formation.

17. One set of sterile flasks after comtamination with natural soil bore fewer perithecia than sterile checks.

18. Artificially-infected wheat seedlings did not develop perithecia in the greenhouse when planted in association with unsterile wheat stubble or when grown in sterile soil watered with a suspension of natural soil.

19. The necessity of biotin for growth and of thiamin for further vegatative development of O. graminis was confirmed. A concentration of 100 µg. of biotin and 10,000 µg. of thiamin per litre of basic nutrient soluation proved optimum for mycelial development.

20. In these studies high concentrations of biotin inhibited growth of O. graminis, a phenomenon not previously reported.

21. It was shown that aspartic acid could be substituted for biotin as a growth factor for O. graminis.

22. The addition of niacin, riboflavin, calcium pantothenate, pyridoxine, folic acid, p-amino benzoic acid, inositol, and vitamin C, singly and in combination, to the basic nutrient solution containing biotin and thiamin in optimum concentrations had no obvious effect on growth.

23. The diluting of nutrients while holding vitamin concentration constant greatly reduced the amount of growth.

24. Of several carbohydrates tested, maltose was the best source for growth and inulin the poorest. Dextrose at the rate of 2% was optimum for myceliul development but concentrations over a wide range were satisfactory.

25. *O. graminis* utilized certain nitrogen sources for growth much better than others. Peptone and asparagine were the best sources.

26. D-glutamic acid was more effective than 12 other amino acids supplied singly or in a mixture. $NaNO_3$ induced induced more growth than several of these amino acids.

27. Growth was not affected by change (before autoclaving) of pH from 3.1 - 9.0 when the organism was cultured on potato dextrose agar.

28. In all of the nutritional studies in pure culture, mature perithecia did not develop although cultures were incubated for 8 weeks under conditions of high humidity and relatively cool temperature in the natural light of the greenhouse.

29. *O. graminis* is specific in its nutritional requirements for the production of perithecia-like black bodies. Biotin and thiamin are both required; no bodies developed below a concentration of 1.0 µg. of biotin per litre. Pyridoxine, folic acid and p-amino benzoic acid seemed to increase their production slightly.

30. Perithecia-like bodies did not develop in agar culture when a high vitamin-nutrient ratio was maintained.

31. When growth factors were supplied as wheat-stem extract, a 5% concentration of dextrose was required for black body formation; but 2% was effective when biotin and thiamin were supplied in a highly purified form.

32. Of 17 nitrogen sources tested, only $NaNO_3$ and dl-serine induced the production of perithecia-like bodies.

33. In general, these studies indicate that although several factors influence the degree of fruiting of O. graminis a proper balance between these factors is probably required for the initiation of fruiting.

ACKNOWLEDGEMENTS

The writer wishes to express his thanks to Dr. A. W. Henry, under whose direction the work was carried out, for helpful suggestions and criticisms during the progress of this investigation; to Mr. E. W. Burton for preparation of the figures; and to the National Research Council for financial assistance.

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